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THE BOTANICAL REVIEW

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RECENT ADVANCES IN THE STUDY OF CHROMOSOME STRUCTURE

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INTRODUCTION

Considerable advances have been made in the study of the structure and chemistry of chromosomes within recent years, advances that have been promoted largely by improved techniques previously reviewed (164, 166, 192). The results suggest the need for a reappraisal of our ideas. One of the most important facts emerging from these findings is that the chromosome structure of plants is not chemically identical with that of animals. In addition, concepts concerning the physical and chemical nature of genes also need critical re-evaluation. Investigations on the physical nature of chromosome structure have been excellently reviewed by Kaufmann (72). In the present resumé, therefore, attempts have been made toward a critical appraisal of the principal literature only on the chemical nature of chromosomes, and their physical constitution is considered only in the light of the latest findings.

A later article will be devoted to the importance of chromosome alterations in speciation.

PHYSICAL NATURE OF CHROMOSOMES

Chromosome Fibre

That chromosome structure undergoes physical and chemical changes throughout the divisional cycle is well established. These changes in relation to phases of division have been reviewed (72). The permanence of the ultimate structure of chromosomes is undeniable, considering the fact that their individuality and stability are maintained in every generation through unaltered persistence from one cell cycle to another.

The literature demonstrating the permanence of the chromosome thread throughout the divisional cycle, specially through the so-called 'Resting Nucleus' stage, has accumulated recently. The existence of chromatin masses in the blood cells of the white series has been demonstrated under a phase contrast lens (21a). Other researches (e.g., 131a, 209a) show that there are chromosomes of definite shape in the metabolic nucleus. Barigozzi (9a) has made a comparative survey of the work on this aspect and has shown clearly that the resting living nucleus contains regular demonstrable chromosomes with linear differentiation in their skeletons. This ultimate skeleton, with genes as its constituents, is responsible for hereditary stability. It is endowed with certain properties which are exhibited by the changes in chromosome structure during the divisional cycle.

Despite conflicting views regarding the nature of this ultimate structure, there is not much controversy respecting its fibrous or thread-like constitution. The permanence of this thread has been well established through the researches of a number of workers (vide review by Kaufmann, 1948), including the most critical method adopted by Zirkle (210). Ris (148), while supporting the chromonema concept, adheres to the idea that chromomeres contain the same fibrils as interchromomeric segments and that the two differ mainly in the arrangement of constituent fibrils. The alveolar theory (80) is now absolutely discarded. The opposing views, postulating a chromomeric (10, 34, 41, 64, 68, 71, 84, 98, 99, 133) and a chromonemic (3a, 32, 81a, 96, 97, 101, 107, 142, 186, 189) nature of the thread, may be reconciled under a common principle. The apparently homogeneous chromonemic appearance of the thread, in certain cases, may represent chromosome structure at the early divisional stage, whereas the chromomeres appear with the gradual deposition of nucleic acid or nucleoprotein at the gene sites. In others, the chromomeric appearance may exist even earlier, depending on the time of initiation of nucleic acid or nucleoprotein synthesis by the genes and the quantity synthesized. However, it is clear that the thread is microscopically homogeneous during certain stages and that the heterogeneity comes in certain phases of division. This apparent homogeneity in certain stages, in most species, does not present a true picture. It is mainly due to the limitations of the techniques used, which do not permit minute distinction between genic and non-genic sites in normal plants and animals to be brought out, under

the present resolving power of the microscope. Though appearing homogeneous in a number of cases, the thread must fundamentally be heterogeneous, even at its ultimate level, because of the presence of active and inactive regions.

McClintock (117) has claimed that genetic effect is associated with deficiencies at the interchromomeric regions. This has, however, been taken by Ris (148) to suggest that genetic stability may not always be attributed to chromomeres alone. In this connection it may be suggested that deletions of interchromomeric regions may affect the position balance maintained between the genes. This may imply that heterochromomeric regions may have some indirect effect in maintaining gene stability, not being themselves involved in gene constitution.

This fundamental thread has been amply demonstrated to be capable of undergoing spiralization (106), though the cycle of spiralization of all of its segments may not be the same. This process is especially responsible for changes in the size of chromosomes during different phases of division. The means by which this spiralization brings out certain differences in different chromosome parts is discussed later.

The nature of the fibrous structure which constitutes this ultimate skeleton has been debated at length. In recent years a number of authors (4, 5, 6, 12, 13, 113) have claimed that chromosome fibrils are composed of end-to-end accumulations of rod-like particles. It has been fully illustrated by different authors that the thread is composed of many fibres (1, 55, 104, 105, 136, 177). The evidence cited and its limitations need not be further discussed here, since it has been thoroughly discussed by a number of previous workers (55, 72, 87).

Lately, advantage has been taken of chromosomal abnormalities induced by X-rays, ultra-violet rays, etc. in interpreting the multiple nature of chromosomes. In cells arising after meiosis, a number of workers (81) have shown that breaks can be induced which affect only half of the chromatid, thus suggesting at least a quadristranded nature of chromosomes. That each chromosome is composed of a number of separable units has been shown (66) in experiments on the detection of mutations in successive generations of meal moth following irradiation.

Radioactive substances also have been successfully used in the study of chromosome structure. Application of tagged P^{32} to amoebae and subsequent determination of lethal effects in successive generations

induced the suggestion (51) that the chromosomes are composed of at least 32 or 64 units.

Use of radioautography in this aspect has been pointed out (76). Chromosomes treated with radioactive material can be placed in contact with a thin photographic film, and the presence of radioactive materials in the subunits can be studied after developing the film. Taylor and others (189) have recently published interesting observations following the use of radioactive substances. The process involves application of Tritium H^3-tagged thymidine to roots, which becomes incorporated in the DNA formed in the strands. Transference of the roots to colchicine next, blocks anaphase movement of the chromosomes. Presence of radioactive DNA has been recorded in metaphase of first division. But in second mitotic division following treatment, radioactive element has been recorded only in one of each pair of sister chromatids, indicating that radioactive isotope was incorporated only in the new half chromatids during chromatid division. This result has been taken to suggest that there was a longitudinal split in the chromosome during its entrance to interphase.

Lately, a number of workers have demonstrated with the aid of electron microscopes (53, 54, 80, 89, 143, 144, 145, 195, 207, 208) that the fibre is composed of innumerable lamellae. Obviously, as one gradually approaches the molecular level through refined techniques, especially involving electron microscopy, the degree of lamellation can be recorded. Gay (57) records fibrils 200Å-500Å thick in her sections of salivary chromosomes of *Drosophila.* Microfibrils of 100Å-200Å thickness have been noted in sections of carp erythrocyte nuclei (209). Chromosome fibrils appearing as rods of 500Å thickness have been recorded (145, 147) in sections of isolated lampbrush and meiotic prophase chromosomes of amphibia. The fibrils have been shown to be double, being made of two closely associated fibrils of 200 Å thickness. In interphase nuclei, mass of fibrils about 200Å thick has been noted by Ris.

According to Lowman's (100a) electron microscope observation of salivary gland chromosomes, chromosomes contain nucleoprotein in the form of granules 200-300Å in diameter. Protein strands connecting the granules are approximately 100 Å in diameter. The intergranular strands in the banded areas are 100 Å and in interband areas 700-900 Å in length.

Kaufmann (76), from his observations of electron micrographs of ultrathin sections of somatic prophase chromosome, contends that the smallest clearly defined fibrillar components are about 125Å thick, which in end view appear as rings. The wall of this ring is about 40Å thick and surrounds a central core of 40Å thickness. The dimensions of the smallest discernible chromosomal constituent noted by him is very near the dimension of the DNA molecule as stated by Watson (202) and Watson and Crick (203). Kaufmann (76) further notes that there are at least 64 separable strands in them and that there may be as many as 128.

In the light of these findings it seems that the conflicting reports about the nature and number of fibrous units, by different authors, are due to the degree of lamellation they could record in their materials with the available techniques. Any statement regarding the precise details of lamellation, based on the methods at present employed, which are limited in application, is bound to be erroneous. Much more improved techniques are required, specially with more advances in the study of electron diffraction patterns, to assert with certainty the degree of lamellation in the chromosomes of a species. Therefore, considering the lamellar nature of the fibre to be an accepted fact, the only definite statement that can be made at present is that, irrespective of the number of lamellae which compose a chromosome, they behave as a binary unit during division. Such behaviour is fundamentally necessary for maintaining the stability and permanence of chromosome structure. Acceptance of a lamellar nature calls for a revaluation of the theory of precocity, as suggested by Darlington (39), to account for the initiation of meiosis. Future researches may indicate how far the theory of precocity—doubtless a very reasonable suggestion, but based on speculation by Darlington— can be reconciled with the multiple lamellar nature of chromosome fibre, so clearly demonstrated in recent years.

Difference Between Heterochromatic and Euchromatic Regions

Chromosomes, in general, are composed of two complexes — heterochromatin and euchromatin. While euchromatin constitutes the major part, heterochromatin is chiefly present on both sides of the centromere, at the telomeres and secondary constrictions, and also, in a number of cases, at the intercalary segments (197). Since none of the special characteristics attributed to heterochromatin by different authors

has been found to be universally applicable to all heterochromatin, Vanderlyn's (197) interpretation of the morphological distinction between heterochromatin and euchromatin seems to be the most plausible. As neither alloycycly nor the property of remaining condensed or not condensed throughout the divisional cycle is shown by all heterochromatic segments, it is better to consider that any segment displaying properties different from those of euchromatin should be termed "heterochromatin," as suggested by Vanderlyn (197). Such an idea, however, is purely relative and distinguishes heterochromatin only in relation to euchromatin.

The above statement does not necessarily imply that all heterochromatic regions, including those of the primary and secondary constriction regions, are fundamentally alike and that in their behaviour they represent only one or more phases in the allocyclic nature of heterochromatic segments. It is evident that the term "heterochromatin" covers a rather heterogeneous assemblage of complexes represented by their diverse characteristics in various species.

Although much controversy exists regarding the function of heterochromatin, it is clear that this term, "heterochromatin," encompasses a variety of structures and that different kinds of heterochromatin are present in chromosomes. It is yet to be seen how far the term is applicable to the secondary constrictions of chromosomes, which are responsible for the organization of the nucleolus. This problem is discussed later in this review.

In addition to previous works, a number of authors have lately shown that the degree of heteropycnosis is not constant in heterochromatic segments (46, 97, 176). It has been claimed (97, 176) that heteropycnosis is dependent on the thickness of the gyres, the double helical nature or the nature of packing. A number of recent authors (101, 124, 142) have noted coils of chromonema in the interphase staining blocks. According to Lowman's (100a) observations, strand material in the salivary gland chromosome is either absent or reduced in heterochromatin.

There is no denial that a chromosome thread is continuous throughout euchromatin and heterochromatin. As regards the differential nature of the two segments, Ris (148), from his studies of electron micrographs, contends that heterochromatic fibrils are 200Å wide,

as are those of euchromatin, but that the degree of packing is different in both.

It has recently been noted by Hannah (62) that more than one kind of heterochromatin may be present in a chromosome. Four types have been distinguished in the salivary chromosome of *Drosophila.* Attention has lately been drawn (75) to the fact that, though breakage sensitivity to radiation may be the same for all segments, a qualitative difference of breakage, with regard to subsequent behaviour of breakage ends, may be present. It is therefore not unlikely, as suggested by some workers (204), that all intermediate types of heterochromatin between active and inert can be observed. In order to work out this aspect, the necessity of a functional study of different genetic loci has been emphasized (156, 157).

Literature on the function of heterochromatin, too, has accumulated in recent years (62, 156, 157), which indicates the confusing state of affairs. Though some specialized cellular function (197) has been attributed to heterochromatin, no definite data in this direction are available. Some authors (108, 133) assert its polygenic nature, attributing to it supplementary effects controlling continuous variation, whereas others (62, 156, 157) consider that it is primarily concerned with chromosomal metabolism. Gay (57) attributes to heterochromatin a function facilitating cytoplasmic synthesis. This confirms the original idea as to its role in protein synthesis.

Centromeres

The nature of the centromere has been an issue of considerable controversy (151), and ideas regarding it have remained at a speculative level. Pertinent data gathered recently are fundamentally different from those of previous workers. While Darlington (36), Nebel (125) and others visualize a compound structure, suggesting a liquid body containing micelles oriented parallel to the long axis of the chromosome, Schrader (152) assumes a spherular constitution. Carothers (23) considered this portion to be constituted of a temporarily modified chromomere. Matsuura (109) regards this region as a specialized part of the genonema. The merits and demerits of a number of theories have been discussed by previous workers (72). Recent observations, however, need comment. Tjio and Levan (193, 194) and Lima-de-Faria (93, 94, 95), with the aid of oxyquinoline tech-

nique, have demonstrated under the microscope the structure of the centromere in plants in which its position is localized. It has been shown that in each of the two metaphase chromatids, in the primary constriction region, two chromomeres, joined by a thread, form the central structure. These two chromomeres are in turn joined, respectively, with the two arms of the chromosome by fine threads. As the two chromatids lie fully adpressed, the chromomeres appear to be quadruple structures, in which each part is a mirror image of the others. The entire structure therefore consists of three parts: *a*) the thread between the centromeric chromomeres and the arm; *b*) the interchromomeric threads; and *c*) the chromomeres.

Such a quadruple structure, as demonstrated by Tjio and Levan, can easily explain the origin of isochromosomes (36) and chromosomes with terminal centromeres through transverse breaks. As this special type of chromosomes obviously possesses, at the initial stage, at least two centromeric chromomeres instead of four, they can organize themselves on the spindle, and survive with little difficulty. An exceptional case was reported in *Zea* (138) where an arm with a terminal centromere was gradually lost during division. In *Phleum echinatum* (144) chromosomes with terminal centromeres have been demonstrated, in which the latter consist of two chromomeres in the metaphase stage instead of four. Two other workers (30, 100) have also reported terminal centromeres. Survival of isochromosomes remains rather problematic in view of previous theories (36) but can be easily accounted for by the quadruple theory of the centromere.

In addition to the localized type of centromere, a number of workers (22, 26, 102, 103, 126, 127) have worked out the presence, and demonstrated in detail the behaviour, of diffuse centromeres in species of *Luzula,* a genus under the family Juncaceae. Though there is a difference of opinion as to whether the chromosomes of this genus should be regarded as "polycentric" (88) or as having "diffuse" centromeres, the centromeres are different from the common localized type. The parallel movement of the sister chromatids along the spindle, instead of being attached at a particular point, during anaphase, is considered strong evidence of their diffuse nature. During meiosis, the first division is equatorial for such chromosomes and not reductional. It has further been claimed that increase in chromosome number in this genus is mainly effected through fragmentation, a conten-

tion which has been proved by survival of X-ray-induced fragments (27). The centromeric property, being diffused, is present in each fragment and thus enables it to survive. A similar type of centromere and its behaviour are reported by some workers (58, 67, 82, 128, 131, 141, 201) in a number of plants and animals. Piza (131), however, states that in *Tityus* the two ends of chromosomes are provided with centromeres, which seems debatable in view of the works of other authors (140). In addition, it is well known (204) that there are polycentric chromosomes in *Ascaris*. The terms "polycentric" and "diffuse" seem to depend mainly on the degree of aggregation (150, 151). Recently, in the moss *Pleurozium schreberi*, Vaarama (196) noted a type, termed by him "semilocalized centromere," in chromosomes characterized by frequent exchanges in the location of kinetic activity in meiosis.

Highly interesting observations have been presented (70, 129, 134, 139, 140, 200) of minor centromeres in addition to normal ones in plants. In the "neo centromeres" (140), block ends provided with the centromeric function are associated with a segment of heterochromatin. Rhoades and Kerr have distinctly shown that in the absence of the main centromere, active mobility is not noticed.

No ideas have yet been put forward regarding the nature of the thread in the centromeric region. It is undeniably a specialized region of the ultimate protein skeleton of the chromosome. Following Feulgen reaction and staining with basic dyes, the centromeric thread and the chromomeres behave positively, whereas the remaining parts show no reaction whatsoever (93, 94). Two fine stained threads with minute chromomeres are barely visible in critically prepared slides. The question therefore arises as to whether there is any enveloping substance, and if so, what is its nature?

Formerly it was generally believed (197) that the regions on both sides of the centromere are heterochromatic; they often appear as condensed blocks of "prochromosomes" during the resting stage and remain destained during metaphase, showing typical allocyclic behaviour. On the basis of the recent findings by Tjio and Levan (193, 194), it seems clear that the destained appearance of the primary constriction region, except the centromeric structure in well-clarified preparations, is attributable to the envelope which forms the major part of the constriction region. The presence of an envelope is shown by its

staining interphase. If the term "heterochromatin" is applied to the destained substance constituting this envelope, it must be borne in mind that this substance does not go into the composition of the main thread of the centromeric region, which is Feulgen-positive during metaphase. It forms only an envelope and is not to be considered as constituting the ends of the centromere, as previously believed. This revision of ideas should be taken into account while defining the nature of the primary constriction at metaphase.

Under the usual methods of fixation, without any special treatment with oxyquinoline (194), coumarin (170), p-dichlorobenzene (171), etc., the primary constriction appears as a clear gap with no evidence of any thread within its body, even in metaphase plates, where this region is very pronounced. This implies that the thread is not stained by those methods of fixation. It is not unlikely that the enveloping substance remains as a compact mass which does not allow penetration of the staining fluid to the inner skeleton. With certain reagents, e.g., oxyquinoline and coumarin, this mass possibly becomes loosened and stretched, and the thread, too, shows the staining reaction.

Spiralization in the centromeric thread is another issue which has not received much attention so far. As the centromeric gap does not show much change in length during the divisional cycle, it is clear that it does not undergo marked spiralization and despiralization. But after treatment with oxyquinoline or other pretreatment chemicals, the centromere regions appear to be somewhat stretched. This slight stretching may be due to two reasons: *a*) the protein thread, though unspiralized, may remain slightly folded or crumpled in metaphase, at least to a certain extent, and may become stretched after treatment due to loosening of the cementing envelope; or *b*) the stretching of the folds may be caused by the contraction undergone by the two arms of the chromosome on the two sides of the centromere. Of the two alternatives, the latter seems more plausible, as the contraction of the two arms following pre-treatment is nearly universal.

A summary of the present knowledge regarding the centromere shows that:

a) The centromere may be any one of five types: localized, semilocalized, diffuse, polycentric, or a neo centromere.

b) The metaphase centromere of the localized type is composed of:

1) Two chromomeres in each thread (having a quadruple nature at metaphase), each being the mirror image of the other.

2) Interchromomeric threads.

3) Connecting thread between the chromomeres and the chromosomes. The latter two are segments of the main protein skeleton. The entire structure is Feulgen-positive.

c) There is a surrounding envelope of a substance, not staining in metaphase, which may be heterochromatic. No heterochromatin is present on the two sides of the centromere, as was previously thought.

d) The continuous thread in the centromere region remains slightly folded and becomes stretched in preparations following pretreatments.

There is considerable difference of opinion regarding the split or unsplit nature of centromeres in mitotic metaphase (vide 49b, 128a, 92a, 95 and 117a). According to Davies (42a), the two theories can be reconciled on the basis of the observed fact that the centromeres behave differently under different metabolic conditions. Abnormal behaviour has been noted when nucleic acid metabolism has been upset. A majority of them have unsplit centromeres, whereas the split condition in others is due to inherent difference in metabolic activity or to the influence of narcotics.

Secondary Constrictions of Chromosomes

In addition to primary constrictions, certain other hiata are also noticed in some chromosomes. They are generally referred to as "secondary constrictions." This term has been applied to a number of chromosome segments which differ widely in function (72).

Secondary constrictions, according to Gates (56) and others, are those gaps or regions in chromosomes, the functions of which are directly concerned with the organization of a nucleolus. They represent the loci of nucleolar origin, and nucleoli have actually been shown (65) to form in these segments in *Vicia faba.* On the basis of this assumption, there is not much difference between satellite constrictions and secondary constrictions. When the nucleolar segment of a chromosome lies at the distal end and the chromatic body at the end is extremely minute, the term "satellite" is often used. When the segments are intercalary, they are generally referred to as "secondary constrictions." The thread connecting the satellite with the chromosome arm, called the "satellite stalk," may not necessarily

be visible in all cases. For example, in *Vicia faba* (65) the satellite stalk is distinctly Feulgen-positive, whereas in *Hordeum vulgare* (172) and some other species it is not stainable. Similarly, in the secondary constriction regions of *Polygonatum* (184) the thread in the segment is clear, but in a majority of other plants it is not visible. It is well known that the stainability of the thread is due to its nucleic acid content. In cases where it is not visible, the staining may be too faint to be resolved under the microscope. The presence of a spiral structure in these zones has been claimed by some authors (137, 184) because of their contractions under cold treatment, general appearance and width. The problem, therefore, remains as to whether the visibility or non-visibility of these threads is due to the quantity of stainable material in them, or whether this behaviour is a reflection of their qualitative difference. It is also not unlikely that the non-staining envelope in the constriction segment, demonstrated to be of phospholipide nature (2, 135), may differ in the degree of compactness in the two types of constrictions. The less compact they are, the more stainable the inner thread will be, due to access of the staining fluid to the protein skeleton. These aspects have to be investigated before any qualitative difference between these two apparently different types of constrictions can be claimed.

The substance responsible for the origin of nucleoli has been termed "nucleolar organizer" by McClintock (116). In *Zea Mays* it has been located at the base of the satellite stalk. Klingstedt (83) has opined that nucleolar organizer is a special kind of heterochromite, the productive activity of which is postponed until telophase. There is difference of opinion regarding the location of this organizer in different species (137, 184). Whether nucleolar organizer is present in all species remains to be determined.

Darlington and La Cour (38, 40) have shown that, following cold treatment, certain heterochromatic segments become very much pronounced. Their low stainability after cold treatment, according to these authors, causes them to simulate secondary constrictions. Those investigators have referred to these regions, starved of nucleic acid in cold, as "secondary constrictions." Resende (137) suggests that these starved areas of Darlington and La Cour are all secondary constrictions.

Darlington and La Cour regard all secondary constrictions as representing merely a phase in the allocyclic nature of heterochromatic

segments. They do not regard the correlation between secondary constrictions and nucleoli as imperative, and consider that nucleoli may originate even in the absence of secondary constrictions and satellites. This idea has, however, been questioned by a number of workers (14, 15, 28, 162) who have shown that nucleoli are always associated with secondary constrictions or satellites. The observations of Darlington and La Cour imply that these differ from primary constrictions only in the absence of a centromere. It appears to us, due to reasons mentioned below, that the secondary constriction regions, responsible for the formation of nucleoli, are segments differentiated from the rest of the chromosome.

The heterochromatin in the vicinity of the primary constrictions generally displays allocyclic behaviour, being destained during metaphase and brightly stained in interphase. Telomeres show allocyclic behaviour sometimes or remain condensed throughout. But the secondary constriction segments of all species, wherever they are present, remained destained, appearing as clear gaps throughout the divisional cycle and never stain, even at interphase. If the staining behaviour is a reflection of their chemical nature, then the heterochromatin of the secondary constriction regions is chemically differentiated from the rest of the heterochromatic segments.

In addition, the segments designated as secondary constrictions by Gates and others (14, 15, 28, 56, 162a) are directly concerned with the production of nucleoli, whereas other heterochromatic regions are not endowed with this property. Caspersson (24) has proposed the term "nucleolus associated chromatin" for the constituents of these regions.

Another line of evidence has been derived from the trichloracetic acid treatment of chromosomes. It has been shown (163) that, following treatment in this acid at dilute concentration at a low temperature for a specific period, the heterochromatin of the primary constrictions responds positively to Feulgen staining, while that of the secondary constriction regions does not. Similarly, after treatment with mercuric nitrate (91) for a particular period, the primary constriction regions react positively with the basic dyes, while the reaction is negative at the secondary constriction segments.

From all this evidence it seems clear that the secondary constrictions, responsible for the origin of nucleoli, have fixed positions in

the chromosomes of a species and are distinctly differentiated segments, different from the other constriction regions. They do not require any treatment in cold temperature to bring out their identity. Low temperature may in certain cases exaggerate their appearance. The term "heterochromatin" may be loosely applied to the substance in the secondary constriction regions in the sense that it is different from euchromatin. "Nucleolar heterochromatin" is a more suitable term for this substance. The "nucleic acid starved" areas of Darlington are quite different from nucleolar segments, and the heterochromatin in them is possibly identical with that in the rest of the chromosome.

The last issue to be discussed is the nature of spiralization, if any, present in the thread of the secondary constriction segment, responsible for the formation of nucleoli. Usually these segments remain practically the same in length throughout the divisional cycle, and as such they give no indication of their physical state. Suomalainen (184), Resende (137) and others have, however, visualized a spiral nature in these segments, especially in relation to their inconstancy in size in a number of cases. But recently, improved methods involving pretreatment chemicals have helped in observing the normal state of the thread in the chromosome. After these treatments, the secondary constriction regions become so widely separated from the chromosome body that the end segments often resemble fragments. That they do not become detached is proved by the continuity of the protein thread, which can be demonstrated by a protein test (unpublished observations in this laboratory of the authors). Such behaviour obviously implies that, under normal conditions, the thread remains highly spiralized and condensed, whereas it uncoils in smears following pretreatments. This further suggests that the enveloping substance (phospholipide of Serra) in the constriction region remains condensed under ordinary methods of fixation. Pretreatment causes a loosening of this mass of cementing material, due to which the thread uncoils, a process facilitated by smearing. It is interesting to note that even under these treatments, the centromeric constrictions do not become significantly pronounced as compared to secondary constrictions, possibly indicating the absence of spirals in that region of the thread. In view of the function performed by the secondary constriction segments, it may be assumed that the spiral of the secondary constriction possibly undergoes uncoiling dur-

ing formation of the nucleoli. Experimental evidence is needed for confirmation of this idea.

The above data also indicate the role played by spiralization in the different aspects of chromosome metabolism. Their presence and absence in different segments of chromosomes, which are distinctly set apart for different functions, suggest clearly that the physical state of the inner thread facilitates, in a direct or indirect way, the functions assigned to them. This issue needs further investigation for clarification.

CHEMICAL NATURE OF CHROMOSOMES

Since the time of Miescher's (118) analysis of sperm nuclei in 1874 and the application of fuchsin sulphurous acid reaction by Feulgen (47) in 1924, considerable advancements have been made in the study of the structure and chemistry of chromosomes. These techniques have been reviewed by Sharma (162b, 164) and their limitations pointed out. Three excellent reviews (35, 42, 160) on the structure and the method of study of nucleic acids have already been published. The structure of the nucleic acid molecule is now well-known, and at least five bases, including 5-methyl cytosine (206), are known to go into its constitution, replacing the four-base tetranucleotide constitution of earlier authors (92). Though the original idea of the nucleoprotein constitution of chromosomes is still accepted, different views have been presented regarding the location of different types of nucleic acids and protein molecules in the chromosome substance. The initial concept of a chromosome being composed of a skeleton of basic protein, with a covering of desoxyribonucleic acid forming the matrix (61), has undergone a complete change. This change has resulted mainly from recent refinements in techniques. These techniques include studies through ultra-violet absorption methods (25, 132, 149), enzyme treatment (21, 73, 74, 77, 114), X-rays (8, 75), polarized light (130), chromatographic analysis (29) and, lastly, the electron microscope (12, 13, 80, 148). There is still considerable controversy about the chemical nature of chromosomes, but the results are indisputable indications of the rapid progress being made.

Complexities in the chemical nature of chromosomes, worked out through the researches of different schools, involve both its nucleic

acid and protein components. Gradually it is becoming apparent that nucleic acids of both ribose and desoxyribose types as well as both acidic and basic proteins enter into chromosome constitution. Quantitative estimation of all the components has been made possible through the photometric methods adopted by Pollister and Ris (132), Leuchtenberger (90) and others based principally on Caspersson's method (24).

The pyronin-methyl green technique (20) has been employed to localize and differentiate the two types of nucleic acids through staining procedure. The green staining of DNA by methyl green and red of RNA by pyronin have been shown (85, 86) to be due to the polymerized nature of the former and the depolymerized structure of the latter. By stoichiometric methods it has actually been shown that one dye molecule of methyl green combines with ten phosphoric groups.

For the detection of DNA alone, a number of methods, both physical and chemical, are in vogue (162b, 164). Chemical methods for its detection involve the localization of its desoxy sugar moiety as well as its phosphoric groups (35, 59, 169). Physical methods are based on the absorption spectra of purine and pyrimidine compounds of the nucleotides at 2600Å (25, 132, 149). Studies based on X-ray and electron diffraction patterns (80, 148), too, have provided an insight of the orientation of DNA molecules in chromosomes. In addition, use of two enzymes, ribonuclease and desoxyribonuclease, has helped to demonstrate RNA and DNA in chromosomes through methods based on digestion of these components by these enzymes in chromosomes. Trichloracetic acid (149a 163, 168) and perchloric acid techniques (45, 161) have been utilised in differential extraction of DNA and RNA, respectively, from chromosomes.

Pepsin and trypsin (73, 74, 79, 114, 175) have been used in detecting the different types of proteins in chromosomes. Trypsin digests proteins of the basic type, while pepsin digests those of the non-basic type. Serra's (158) test has helped in determining the types of amino-acids in the different chromosomes proteins.

None of these methods, however, is free from limitations. Their possibilities and drawbacks have already been reviewed (164). An outline of the observations deduced from one or more of these techniques is given below. For the sake of convenience, the researches

on plant and animal chromosomes have been reviewed separately as certain fundamental differences are noted between them.

Darlington (37), in 1942, presented a chemical picture of the chromosome, based chiefly on Caspersson's observations. According to him, in *Drosophila* the chromomeres of euchromatic regions are composed of thymonucleic acid, histone and the interchromomeric region of globulin. The heterochromatic region, too, shows a high histone content, and thymonucleic acid is also present.

Kaufmann and his associates (77, 79) have mainly centered their studies on enzyme digestion experiments on salivary gland chromosomes of *Drosophila.* Their observations reveal the presence of two separable complexes in chromosomes, one the ribonucleprotein and the other the desoxynucleohistone, both of which are capable of maintaining the chromosome structure. They also suggest that chromosomes consist of an interconnected system of proteins, in which nucleic acids are closely joined. No single protein or nucleic acid, they claim, should be considered as the ultimate structural component. The differential nature of chromosome segments has been further demonstrated through differential swelling in different chromosome parts following trypsin treatment (74).

Kaufmann (76) claims that the heterochromatic block in interphase contains a high amount of RNA. It has further been pointed out that some bands in salivary chromosomes of Diptera contain mostly ribonucleoprotein and others mostly desoxyribonucleoprotein. He suggests a study of the differential chemical nature of heterochromatin on the basis of their response to enzymatic hydrolysis.

Mazia and his co-workers (110, 112, 114) have also based their studies on the salivary gland chromosome of *Drosophila* and other animal chromosomes. They claim the existence of a dual structure in the chromosome, in which a nucleoprotein continuum runs through a non-nucleoprotein continuum in such a fashion that either of them can maintain the structural integrity of the chromosome. Both are responsible for the mechanical properties of the chromosome, and its volume is determined from their joint presence. Mazia (112) has further demonstrated the fibre structure in chromosomes. From observations on artificial protein fibres through the electron microscope, he has contended that the apparent fibrilar structure resolves into a folded sheet, possibly of mono-molecular thickness. Since artificial

fibres and chromosome fibres react similarly to enzyme application, analogy has been attempted between the electron microscope picture of chromosomes and artificial fibres.

Stedman and Stedman have long been launching (178-182) a severe attack on the classical concept of chromosome structure and claim that DNA is not present in any significant amount. They suggest that an acidic protein, named "chromosomin," forms the ultimate skeleton of the chromosome. Recent researches (121, 122) have shown the possibility of the existence of the acidic protein in the ultimate structure of the chromosome, but the absence of DNA in the latter, as stated by Stedman, does not find support in any of the later studies, including those made by the authors (35, 164, 183). All the observations so far recorded unequivocally suggest the presence of DNA in chromosomes. This objection put forward by other workers against Stedman's views has not yet been explained by the latter. Stedman and Stedman suggest that DNA is present in cytoplasm, which takes up colour in Feulgen solution, and that this dye is diffusable and is adsorbed on the chromosome surface. If this claim were correct, the cytoplasm should be coloured in Feulgen staining, which is never the case in critical preparations.

The observations of both Mazia and Kaufmann, as mentioned above, are based on studies on the salivary gland chromosomes of *Drosophila*. Their conclusions are also similar to some extent.

Mirsky (119, 119a) and Mirsky and Ris (121, 122) have, however, arrived at quite different conclusions on the basis of their observations on isolated chromosomes from thymus, pancreas and liver. A new isolation technique has been developed for the extraction of chromatin matter from the cells. The first step involves breaking up the cells in a colloid mill and a Waring mixer. By straining through fine cloth and differential centrifugation, the chromosome mass is then separated from the cellular constituents. Analysis of this chromosome mass shows the presence of both DNA and RNA, and of histone and non-histone proteins. Fractionation with NaCl solution results in the separation of highly viscous matter and small thread-like structures. The viscous solution contains nucleohistones. The small thread-like structure, which remains intact, has all the characteristics of chromosomes with constriction regions. On analysis, they yield RNA and DNA in the proportion of 4:1 and non-histone protein. Acidic medium

brings about a disintegration of this thread-like structure, and the individuality of the chromosomes becomes lost. Mirsky has termed these thread-like structures "residual chromosomes." According to him, residual chromosomes are responsible for the individuality of the chromosome thread, and his observations imply that they represent the ultimate skeleton. He has also shown that the fibrous character of the residual chromosome is due to its protein moiety, whereas that of the mantle of desoxynucleohistone is due to its nucleic acid moiety. The protein of the residual chromosome is called "chromosin" (120), which Stedman claims to be equivalent to his "chromosomin."

The above discussion shows that there is considerable discrepancy among authors. None of the techniques is perfect, in the sense that they are endowed with certain inherent unavoidable limitations which may to some extent account for these discrepancies. It is also desirable that the different techniques, including isolation methods for chromosome analysis, be applied on the same tissue of a common organism, so that a number of errors in the observations may be eliminated. Notwithstanding the controversial issues, a clear fact, emerging from the observations of Kaufmann, Mirsky and Stedman, is that the ultimate skeleton contains an acidic or non-histone protein.

A reconciliation between the pictures of chromosomes obtained through chemical analysis can be made by the cytologist's visualization of chromosome structure. The "residual chromosome" of Mirsky coincides with the chromonema as envisaged by cytologists. This is probably the fundamental structure which undergoes spiralization and other processes during the divisional cycle. An acidic protein, possibly of the tryptophane type, definitely goes in its constitution. On the basis of Mirsky's observation, this structure is not free from nucleic acids. The presence of nucleic acid in the ultimate skeleton, however, is yet to be established from more confirmatory data, gathered especially from the metabolic nucleus in ultra-violet absorption studies.

If the reality of the matrix, as demonstrated by cytologists, is accepted, then it may be regarded as comparable to the desoxynucleohistone of Mirsky. The term "matrix" is applied here to the substance accumulating on the thread during the chromosomal cycle. It is not to be considered as a membrane. Mirsky and Ris, however, do not adhere to the term "matrix" as far as it may be regarded as a membrane. In view of the observations of Kaufmann and Mazia, too, it ap-

pears that not only nucleic acid but proteins also enter into its composition. However, there is considerable disagreement as to which types of nucleic acid and protein are present in its constitution. Kaufmann is of the opinion that this desoxynucleohistone fraction is capable of maintaining the individuality of the chromosome structure, a property not attributed to matrix by other authors. In any case, the presence of a non-basic protein in the skeleton and of protein, in addition to nucleic acid, in the matrix is quite contrary to the concept of earlier authors of chromosome structure as composed of histone and a covering of DNA.

Recently Ris (143-145, 148), from his observations with the electron microscope, confirmed the fibrillar nature of chromosomes and suggests that at least the chromosome fibrils consist of a core of DNA surrounded by a shell of protein. The outer shell has been assumed to be proteinaceous because this part reacts with OsO_4, a property common to proteins. The nucleic acid core is assumed on the basis of the analogy of the structure with tobacco mosaic virus. This idea is certainly interesting but it would remain at a speculative level unless the direct analysis of chromosome fibrils, as suggested by the author, is carried out, as done in tobacco mosaic virus particles.

The idea of Ris that heterochromatin contains the same basic unit as euchromatin, a fibril 200Å wide, but differs in the degree of packing either in the system of coils or perhaps in a less regular fashion, needs more data for confirmation. Ris has also questioned the reality of the matrix and considers it to be an artifact. He claims that electron micrographs do not show any evidence of a membrane. This issue is indeed worth consideration, and observations from both physical and chemical angles are needed for clarification.

Moreover, some insight has also been given (12, 13, 113) as regards the basis of linkage of chromosome fibrils. Chromosome fibrils have been shown to be formed by linear joining of rod-like particles. As such particles appear free on removal of calcium and magnesium, it has been suggested that possibly bivalent cations, like Ca^{++} and Mg^{++}, are responsible for this linkage. Ambrose (4-6), however, suggests that these particles are held together by hydrogen bonds, on the basis of his observations that on treatment with chemicals, which are capable of destroying hydrogen bonds, the particles come out free.

Studies, based on polarised light (130), X-ray (8, 75) and electron

diffraction patterns (12, 80, 148), have raised the analysis of chromosome structure to a macromolecular level, and interest has been directed to the arrangement of nucleic acid and protein in chromosomes at the molecular level. Astbury (7) suggests that the nucleotides are flat and that their spacing along the fibres is 3-4Å apart. The original claim of a tetranucleotide constitution is now considered to be invalid, for Wyatt (206) noted a 5-methyl cytosine base in chromosomes. Astbury (7) records a repeat after eight or 16 nucleotides in his data gathered from X-ray diffraction patterns. Astbury and Bell (8) suggested earlier that nucleic acid chains lie parallel to the long axis of the chromosome, whereas Wrinch (205) held that the arrangement of the proteins is at right angles to the long axis of alignment. Furberg (52) suggested a modification of Astbury's view and claimed that in one type of nucleic acid, a spiral around the base is formed by the sugar and phosphate, and in the other he considered the bases as outermost to the zigzag chain of the backbone. Though controversy exists regarding the arrangement of nucleic acid and protein, the polynucleotide constitution of the former seems to be well established in contrast to the tetranucleotide concept.

From observations by X-ray diffraction and other methods, Watson (202) and Watson and Crick (203) suggest that polynucleotide chains are joined through purine bases, that the dimension of each molecule is 20Å and that a full double helix has a pitch length of 34Å. In the preceding part of this review it has already been pointed out, while discussing the physical structure of chromosomes, that evidence concerning the multifibrillar nature of chromosomes and their parallel alignment on the long axis, which is more or less established now, has mainly been derived from X-ray and electron diffraction patterns.

Compared to animal chromosomes, very little data (79) on the chemical nature of plant chromosomes were available till recently. This was mainly due to a lack of suitable materials for such studies. In plants, no structure is known comparable to the salivary gland chromosome of *Drosophila,* which provides an ideal material for analysis.

Lately, considerable work on the chemical nature of plant chromosomes has been carried out by the writers and their co-workers (17, 163, 168, 169, 173-175). Their methods involve enzyme digestion

experiments, use of trichloracetic and perchloric acids, detection of both acid and alkaline phosphatase activity and pyroninmethyl green staining, and also the Serra protein test (158). It has been noted (175) that the best representation of chromosome structure, in terms of phosphatase activity, can be obtained after fixation, in oxyquinoline, phenol and chromic-formalin mixture. After fixation, the different tests mentioned above have been applied on root-tips. The phosphatase cycles of both mitotic and meiotic chromosomes have been studied, and the latter has been found to correspond with the nucleination cycle of chromosomes.

On the basis of these observations it is suggested that the main thread, i.e., the ultimate skeleton of the plant chromosome, is composed of a non-basic protein which survives even after trypsin and trichloracetic acid treatment but not after pepsin digestion. This thread shows intense phosphatase activity. Discontinuous segments of basic protein lie over this ultimate fibre, probably associated in the heterochromatic regions which also show high phosphatase activity. The final envelope is the nucleic acid. The threads in the primary and secondary constriction regions are highly phosphatase-positive. Kaufmann, too, recorded non-basic protein in chromosomes (79), and Serra and others (2, 135) reported phospholipide in the secondary constriction regions.

The non-basic protein in the ultimate skeleton of plant chromosomes finds its parallel in animals. The high phosphatase activity in this thread, even when entirely devoid of nucleic acid, poses an interesting problem. A critical investigation is required in order to find out how the constitution of this non-basic protein skeleton differs in plant and animal chromosomes. Further, no evidence of nucleic acid is available in this ultimate skeleton, as found in animals. Lastly, in plants the histone is not present as a continuum over this ultimate thread, but is found only in patches, contrary to the situation in animals. The presence of both types of proteins in the heterochromatic regions is specially significant in view of the function attributed to these specialized chromosome segments. The report of Serra and others of the presence of phospholipide in secondary constriction regions is also significant with regard to its function of nucleolar organization. This observation distinctly proves that they are specialized segments set apart in correlation to nucleolar production.

In view of all these observations, it is quite clear that data gathered from the salivary gland chromosomes of *Drosophila* cannot be applied to the chromosomes of plants. In animals, too, generalizations must not be based on the salivary gland chromosomes, since there are certain differences between these chromosomes and those of the thymus and other organs. Besides this, plant chromosomes differ from those of animals in some fundamental aspects as well. These records show the need of a separate critical investigation of the chemical nature of chromosomes, both in situ and in vivo, of both plants and animals.

GENE STRUCTURE IN RELATION TO THEORIES REGARDING THE CHEMICAL NATURE OF CHROMOSOMES

Extensive microscopic and genetic studies on *Drosophila* reveal that genes are located the chromomeres, or band regions, of chromosomes. McClintock (117) has, however, claimed that deficiencies in interchromomeric regions cause phenotypic changes—an observation which, according to Ris (148), goes against the original idea of localizing genes in chromomeric regions. Such phenotypic changes, due to the deficiency of interchromomeric regions, may easily lead to an upset of the position balance maintained between the chromomeres and interchromomeric segments in chromosomes. The latter, being highly organized stable units, are supposed to maintain a state of equilibrium between all segments, a disbalance of which may easily cause a phenotypic change. Therefore, the observation of McClintock does not imply that genes are located in the interchromomeric regions. At the present state of our knowledge, there is no valid reason to refute the theory that genes are located in the chromomeric regions.

Chemical analysis of chromosome structure has helped considerably in the elucidation of the nature of genes. As yet, the structure of genes has not been fully analyzed, but certain direct observations have modified the view already existing as to the chemical nature of the gene. The exact basis of specificity is, however, still unknown. Theories accounting for this specificity have been based mainly on the concept that the chromosome is composed of a simple polypeptide protein chain which accumulates nucleic acid over its active regions. The theories fall into three categories according to whether they attribute the specificity to the nucleic acids, to the proteins or to the nucleo-

proteins as a whole, that is, to one of the components or to the entire compound.

Authors (50) who do not regard the nucleic acid as the agent responsible for specificity are of the opinion that the transitory appearance of the nucleic acid alone suggests that it cannot enter into the composition of the genes. The chemical composition of genes must have permanent stability in order to exert control over genetic characters. Besides this, the uniform chemical constitution of the nucleic acid, with a maximum of five bases, sugar and phosphoric acid, cannot account for gene-to-gene differences. According to the proponents of this theory, proteins, with their large variety of well-known amino acids going into the formation of the polypeptide chain, can easily account for gene-to-gene differences, as visualized by geneticists, An active group can be held to be present in each gene locus. Frey-Wyssling (50) holds that the operative groups in the active nucleus need screening-off during divisional stages. This screening-off of a gene or of specific groups of genes is provided by nucleic acid, which is accordingly distributed in specific areas. A passive role in heredity is thus attributed by him to nucleic acid.

However, a salient point in this connection is that there is no experimental proof to indicate the transitory nature of nucleic acid, that is, nucleic acid is absent from interphase chromosomes. Its non-detection may be due to the highly hydrated nature of chromosomes in that stage.

That desoxyribonucleic acid enters into gene composition has been claimed by a number of workers (18, 111, 115, 119, 123, 198, 199). A proportionate increase in the DNA content of haploid, diploid and polyploid cells has been shown. Mirsky and Ris (122, 149), by means of photometric analysis, have also shown that the DNA content remains fixed for the haploid set of chromosomes throughout the divisional cycle and that, as such, this substance is not transitory. They have strongly criticized the earlier method of detecting the staining intensity. Mazia (111 attributes all the hereditary differences between human beings to the minute differences in the molecules of DNA.

Further support of this theory is obtained from the observations of Chargaff (29) and his associates following chromatographic methods. They (29) have shown that the DNA fraction in every tissue of an organism remains fixed throughout, whereas for different organisms

it varies. The proportion of RNA content in chromosomes has been found to vary during divisional stages (69, 122). It has also been claimed that the bases are not present in equimolar proportions in all, and for different organisms the ratios are different. This observation obviously goes against the original idea that the DNA of every organism is of uniform chemical morphology. It is also noteworthy that Wyatt (206) has recorded the presence of another base, 5-methyl cytosine, in the DNA extracted from wheat germ and calf thymus; the amount in the two has been found to vary.

In support of this theory and objecting to the idea that proteins form a stable unit in all cells, certain workers (33) show that fractions of histone and protamine, some of the principal components of chromosomes of salmon and fowl, vary in different tissues of the same organism. In any case, all these observations indicate that DNA neither is transitory in appearance nor possesses a uniform chemical structure in all organisms. A variety of forms can easily be visualized now which can also satisfy the requirements of a geneticist. It has also been implied that there are different molecular patterns of DNA in chromosomes. Much more evidence in support of this contention is needed.

The discovery of the principle responsible for the transformation of *Pneumococcus* (3, 9, 43, 60, 115) provides important evidence in support of the theory claiming DNA as the basis of specificity of genes. It has been demonstrated that some uncapsulated Pneumococcal types can be transformed into capsulated ones with the extract of a type containing chiefly DNA (9, 115). The explanation put forward is that self-duplication, resulting in a large amount of the active substance, is the factor underlying this transformation. If, however, DNA solely is responsible for this process, a small quantity of it would be sufficient. Actually, however, large amounts of DNA are needed for this purpose. It seems, therefore, that the role of the small quantity of protein, which has also been found to remain in the extract, is far from negligible.

Emphasizing the importance of DNA in chromosome reproduction, Ris (148) has drawn attention to the fact that the time of DNA synthesis and that of chromosome reproduction always coincide during interphase (188, 191). Ris has also laid emphasis on observations (48, 49, 63, 153) regarding tobacco mosaic virus particles, where

it has been shown that RNA forms a core surrounded by a protein shell, implying a correlation between nucleic acid and the substance responsibility for genetic stability. This observation has been interpreted by Ris as an indication that DNA is not only quantitatively but also qualitatively the same for a species. He (147) has also interpreted the fibrils in his electron micrographs of chromosomes of both plants and animals, as composed of a core of DNA surrounded by a shell of protein, thus implying the importance of DNA in the constitution of the gene. The conclusions of Ris are mainly based on the analogy with tobacco mosaic virus particles and osmium tetroxide precipitation of micro fibrils, whereby the surface becomes more electron scattering than the interior. More confirmatory evidence, specially involving direct analysis of isolated chromosome fibrils, is needed to support his conclusions.

Schultz (154, 155, 157) regards nucleoprotein as gene, drawing analogy especially with respect to specificity, auto-reproduction, similar topography and relation to vital processes. This contradicts the theory (50) which emphasizes that genes are independent structures without the character of nucleoproteins. The objection (50) that nucleoprotein is nearly absent in the reconstituted nucleus at the time when gene activity commences is not based on direct observation (cf. 82a). Data on the basis of Feulgen colorimetry and ultra-violet absorption measurements, which alone can provide direct clues to the presence or absence of nucleoproteins in the reconstituted nucleus, is still lacking. Staining intensity may not be a correct index, as a very small amount may not be detected under the resolving power of the microscope.

Serra (159) asserts that the gene is protein in its simplest form but nucleoprotein while behaving as a reduplicating unit.

If all the data on the chemical aspect of both plant and animal chromosomes are considered together, a clear perspective can be obtained as to the chemical nature of genes and the type of protein composing them. The idea regarding the substance controlling its specificity, however, still remains to some extent speculative.

The observations of both Kaufmann and Mazia (77, 81, 111, 112, 113, 114), mentioned in the preceding part of this review, indicate the presence of nucleic acids, as well as of the two types of proteins, basic and non-basic, in chromosomes. Mirsky and Ris (121) have

demonstrated the presence of non-basic protein in the residual chromosome, which they claim to be the ultimate structure. Stedman's "chromosomin" (178, to 182) of the ultimate skeleton is also an acidic protein, that is, undoubtedly of the non-basic type. Observations by the present author and his associates (168, 169, 175) also indicate that non-basic protein is present in the final structure of chromosomes.

In spite of the fact that all observations underlying these conclusions are based on techniques which are not free from limitations, the presence of an acidic or a non-basic protein in the ultimate skeleton is the common inference in all of them. Keeping this in view, it may safely be assumed that an acidic protein is present as a permanent substance throughout chromosome structure. As the substance which enters into gene composition must have stability, acidic protein, in all likelihood, is present in the genic structure.

The next problem is whether there is any other substance in the gene locus, and if present, what is its nature.

Data regarding the other constituents of the ultimate structure are very meagre. Mirsky (119) claims that nucleic acids and nonbasic protein only are present in the residual chromosomes of the animal organs worked on by him. On the other hand, observations (175) on plant chromosomes show that the heterochromatic regions, in addition to this acidic protein, contain basic proteins of the histone type as well and as such form discontinuous segments over this thread. Over this ultimate skeleton, desoxynucleohistone makes a transitory appearance in the form of a matrix.

Researches on plant chromosomes, therefore, suggest that the euchromatic segments and obviously the euchromatic genes, too, contain acidic proteins as well as nucleic acid. The heterochromatic genes contain proteins of the histone type in addition to these two compounds. The presence of histones in the heterochromatic regions is expected in view of the function they perform, that is, conversion of ribonucleoprotein into desoxyribonucleoprotein. The occurrence of an additional type of protein in these regions obviously facilitates this process, especially in the production of nucleoli which also contain a good amount of basic protein. The role of heterochromatin in the synthesis of ribonucleoprotein is well-known (24). Certain authors (76) have reported high RNA content in heterochromatic and adjoining regions in dividing plant cells. The observation, by the present author (175), of both

types of proteins in the heterochromatic regions is contrary to the belief that only the histone type of protein is present in those regions.

Mirsky and Ris (121) do not record histones in the ultimate skeleton. It is not likely that this absence was due to insufficient heterochromatin in the chromosomes investigated.

A point worth consideration is that very high phosphatase activity has been noted in plant chromosomes in their ultimate skeleton (169, 175). Furthermore, Mirsky has detected ribonucleic acid in the residual chromosomes of animal organs, whereas only desoxyribonucleic acid, and no trace of RNA, has been found in plants (169, 175). All these facts obviously suggest that plant and animal chromosomes may differ in certain fundamentals, namely, the acidic protein in one may not be of the same type as in the other and RNA may be absent from one but present in the other. Clarification of these issues will obviously bear much on the concept of genes. These problems are worth investigation, and in order to clarify them, intensive studies on the chemical nature of different groups of plants and animals should be undertaken.

In view of all the observations outlined above, the nucleoprotein nature of the gene seems probable, in which acidic protein may be considered as one of the components and nucleic acid as the other. As regards the specificity of genes, it is true that diverse forms of amino acids are known, which may easily account for the formation of different gene loci in the polypeptide structure.

Inspite of the fact that some authors, as mentioned above, have reported variation in the content of proteins in different stages of somatic and germ cells of certain animals, one cannot say with certainty that a minimal and fixed amount of protein is not present in all cells of an organism. In all likelihood such constancy is maintained, especially in view of the importance of protein in all vital functions of cells and also in consideration of their capacity to undergo autocatalytic reproduction with the aid of a prosthetic group. There is no doubt that a polypeptide structure enters into the constitution of chromosomes, and if it is stable, then the diversities of genes can be explained, at least partially on the basis of differences in different segments of the polypeptide structure.

While emphasizing the role of proteins in gene specificity, one must not ignore the wealth of data recently accumulated, indicating the

importance of DNA in gene differences. The objection that DNA has uniform chemical structure in all organisms has been completely overruled. On the other hand, evidence has been brought forward claiming that the proportion of bases may vary in different organisms but is fixed for a species. The original idea that it is transitory is also no longer valid. The behaviour of pneumococcal strains in culture further substantiates the validity of the idea that DNA may account for gene specificity.

It seems, therefore, that both components of genes—nucleic acid and proteins—are endowed with ability to control gene specificity. Opinions are sharply divided and cogent evidence has been put forward in support of both theories. It is true that a chromosome is a giant complex molecule made up of several less complex molecules of nucleic acids and proteins, and that all form an integrated structure. It is better to visualize at the present state of our knowledge that the entire compound of the gene, the nucleoprotein as a whole, with its diversities in structure in both of the components, contributes to the structural differences between one gene and another and thus controls their specificity of action. No component alone seems to be responsible for gene differences. Though so much insight has been obtained on gene structure and specificity, it must be admitted that data are still to some extent conflicting and not fully based on uncontroversial evidence. Discrepancies in reports can be avoided by using common materials for study. Precision in methods employed is highly essential in securing uncontroversial data as regards the chemical basis of gene differences. Analysis of such gene differences on a chemical level is a task of vast magnitude and needs utmost precision in genetics, cytology, biochemistry and biophysics. The tremendous progress already attained in techniques for the study of finer details of chromosome structure at a submicroscopic level can not be over-rated. As this line of study is proving to be more and more dynamic, much more perfection is expected within the coming few years. The problem of gene specificity would then no longer remain in its present realm of speciation and a solution will be possible through reconciliation of data secured with adequate technique by the workers in different fields of both biological and physical sciences.

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INTRODUCTION

Ever since the attempt by Cameron (6) in 1880 to supply selenate to plants and thereby replace the sulfur of plant proteins with selenium, many investigators have sought to explain the toxicity of selenium compounds on the basis of such a substitution. Because the two elements are similar, it has been an attractive idea to consider that most organisms are unable to distinguish between them and that, as a result, selenium compounds enter and disrupt sulfur metabolic pathways. However, when biological interrelationships are studied, it is equally important to bear in mind the dissimilarities between the elements, between their analogous series of compounds, and especially between selenium compounds themselves, for many physiological responses depend on the physical and chemical properties of the individual selenium compounds. Some of these differences are sufficiently important to warrant their brief reconsideration in this review.

[1] The survey of the literature pertaining to this review was completed in June, 1957.

The history, geology, biology and chemistry, as well as other aspects of selenium, have been treated thoroughly by Trelease and Beath in their book, "Selenium" (89). This review, therefore, will be restricted primarily to papers that have appeared since 1949. Although considerable applied research has been undertaken to help solve the economic problems that have resulted from selenium toxicity to livestock, the papers treated here are mainly those that bear on the mechanism whereby selenium compounds exert their effects.

PROPERTIES OF SELENIUM COMPOUNDS

Sulfur and selenium, which occur in Group VI of the periodic table with oxygen and tellurium, have related nuclear configurations and electron distributions. A detailed analysis of the physical and chemical properties of these elements is given by Yost and Russell (101). An important feature of the elements sulfur and selenium and their compounds is the existence of analogous series of molecules which are similar in composition and in spatial orientation. Both elemental sulfur and elemental selenium occur in allotropic forms with comparable crystalline structures and solubilities in organic solvents. The vapor states of both elements also show analogous polymeric forms. Compounds such as H_2S and H_2Se, which can be prepared by the same methods, have many physical and structural properties in common, although the selenium analogue is in general more unstable and a stronger reducing agent than H_2S.

The two inorganic selenium ions that have been most widely used in biological studies are selenite ($SeO_3^=$) and selenate ($SeO_4^=$). These are analogues, in some ways, of the sulfite ($SO_3^=$) and sulfate ($SO_4^=$) ions, respectively.

SELENITE. A very important property of selenite, from a biological point of view, is its oxidizing power. The molal free energies of formation of several compounds and ions that contain sulfur and selenium are listed in Table I (derived from Latimer, 43). Selenite and selenious acid have lower values than do their sulfur analogues or selenates. Selenite, therefore, is the stronger oxidizing agent and will be reduced more readily. In fact, exposure of selenium dioxide[2] to the dust particles in air will effect a reduction to elemental, pink selenium, as will

[2] Selenium dioxide is hydrolyzed to selenious acid in the presence of water: $SeO_2 + H_2O \rightleftarrows H_2SeO_3$.

dilute solutions of sulfurous acid. It is therefore apparent that the selenite and sulfite ions are not analogous in their oxidation-reduction properties, even though their chemical composition is similar. The comparative ease with which selenite can be reduced is in contrast to the oxidation of this ion to selenate, for which reaction strong oxidizing agents, e.g., H_2O_2, Na_2O_2 or $KMnO_4$, are required.

The relative instability of the selenite ion should be seriously considered when it is introduced into biological media or biological systems. Frequently the ion is incorporated into organic media for the growth of microorganisms, into animal feeds to be used in toxicity studies, or injected directly into animals. The responses of organisms at times have been attributed to selenite without due consideration being given to a possible reaction of the ion with other components of the medium. That such reactions can occur under physiological conditions has been demonstrated by a number of investigators. Painter (64), in his early review of the selenium problem, pointed out that selenite oxidizes sulfhydryl groups, and he suggested that this might be a cause of selenium toxicity. He gave the following general equations as possible reactions:

$$2RSH + SeO_2 \longrightarrow RS{-}\overset{\overset{O}{\|}}{Se}{-}SR + H_2O$$

$$4RSH + SeO_2 \longrightarrow RS{-}Se{-}SR + RSSR + 2H_2O$$

$$4RSH + SeO_2 \longrightarrow 2RSSR + Se + 2H_2O$$

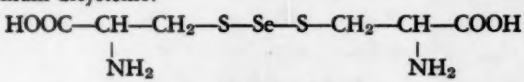
More recently the improved techniques of starch column chromatography and absorption spectroscopy have been applied to this problem

TABLE I

MOLAL FREE ENERGIES OF FORMATION OF SEVERAL SULFUR AND SELENIUM COMPOUNDS AND IONS

	$\Delta F°_{298}$ cal/mole		$\Delta F°_{298}$ cal/mole
$SO_4^=$	−176,100	$SeO_4^=$	−105,120
H_2SO_3(aq)	−128,535	H_2SeO_3(aq)	−101,361
HSO_3^-	−125,905	$HSeO_3^-$	− 97,850
$SO_3^=$	−116,400	$SeO_3^=$	− 87,890
H_2S(aq)	−6,520	H_2Se(aq)	16,720
HS^-	2,950	HSe^-	22,120
$S^=$	23,420	$Se^=$	35,760

in order to determine more specifically the nature of the compounds developed in these oxidation-reduction reactions. Klug and Petersen (41) concluded that cystine is formed by the oxidation of two moles of cysteine, and that, simultaneously, two moles of cysteine are converted into selenium dicysteine:



SELENIUM DICYSTEINE

Similar results were reported by Petersen (67) who found that SeO_2 caused the disappearance of reduced glutathione and the appearance, in a starch column eluate, of oxidized glutathione and of a compound which was tentatively identified as selenium diglutathione (GS — Se — SG). Stekol (87) had earlier interpreted his analytical data to indicate the formation of a selenium tetracysteine.

Little attention has been given to non-enzymatic transformations of selenite in tissues. That the ion can be converted by living animal tissues into elemental selenium, volatile selenium and "bound selenium" of undetermined nature has been demonstrated by Rosenfeld and Beath (77). But autoclaved liver was unable to effect these changes, though an affinity between selenite and the fresh as well as the autoclaved tissue was reported.

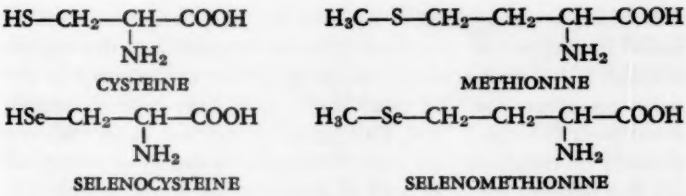
The extent to which toxic effects, frequently attributed to selenite, can be ascribed to the compounds that result from the interaction of selenite with other substances is largely unknown. Some of these compounds may indeed be toxic, since rats can be poisoned by the reaction products of selenium dioxide and reduced glutathione (40).

SELENATE. Selenate is more akin to sulfate than is selenite to sulfite. The physical properties, such as the specific gravity of aqueous solutions of selenic acid (H_2SeO_4), resemble those of sulfuric acid, and the ionizing properties of these two molecular species are practically the same. The two differ in their oxidation potential. Table I shows that the free energy of formation for selenate is much less than for sulfate. Selenates are therefore the stronger oxidizing agents and consequently are more readily reduced than sulfates. A solution of selenate, through which sulfur dioxide is bubbled, can be reduced to elemental selenium but not so readily as the less stable selenite. A non-enzymatic reduction should also be kept in mind as a possible cause

of toxic effects. In this connection, Fels and Cheldelin (17) reported reduction of H_2SeO_4 to elemental selenium by L-cysteine or glutathione when these chemicals were mixed in a culture medium prior to its inoculation with *Saccharomyces cerevisiae*. The acid also reacted with methionine at room temperature to give a substance that contained Se, N and S. The rate of this reaction depended on the concentrations of the starting materials, but the precise experimental conditions were not specified by the authors. Nor have further details been published (Fels, personal communication). The reaction may have its counterpart in the decomposition of methionine by hot 50% sulfuric acid to yield approximately equal amounts of homocystine and methionine methylsulfonium hydrogen sulfate (44).

The structural resemblance between the selenate and sulfate ions is particularly suitable for the interpretation of data in accord with antimetabolite theories as discussed by Woolley (100). In crystals of various salts of these ions, a regular tetrahedral configuration exists, with the sulfur or selenium atoms at the center and the four oxygen atoms at the corners. The distances within the tetrahedra differ. In sulfates the S — O interatomic distance is approximately 1.51 "A", whereas in selenates the Se — O distance is 1.61 "A". Crystals of most sulfates and selenates are isomorphous. These similarities in structure seem to be the basis for the competitive antagonism that has so often been observed between the two ions in growth studies with plants and microorganisms, and have led to the view that selenate prevents normal metabolism of sulfate by competing for the responsible enzymes. Such competition has never been observed between sulfate and selenite and has not been reported for sulfite and selenite to the knowledge of this reviewer. The extent to which biological effects can be attributed either to the structure of selenate or to its participation in non-enzymatic oxidation-reduction reactions can be determined only through the use of suitable experimental criteria.

ORGANIC SELENIUM COMPOUNDS. Many organic selenium compounds have been prepared by methods which are also used to synthesize the sulfur analogues. The chemistry of these compounds has been reviewed by Painter (64) and more recently by Campbell et al. (7). Selenocysteine and especially selenomethionine have received the most attention in biological studies:



In general, the selenium-containing amino acids, particularly selenocysteine, show the same degree of instability as their notoriously unstable sulfur analogues. Information about the chemistry of decomposition of the selenium compounds, critical in work that attempts the isolation of naturally-occurring selenium compounds, has not been as complete, however, as for the sulfur amino acids. Painter (64, p. 204) reported a personal communication from Schaefer and Moxon to the effect that selenocysteine, when boiled in the same concentrations of acids that are used to hydrolyze proteins, slowly decomposed with a separation of elemental selenium from solution. This work has been cited by Whitehead et al. (98) who also state that selenomethionine was stable under similar conditions.

Since many organic sulfur and selenium analogues show essentially the same steric configurations, the selenium-containing amino acids fit quite well into the concept of a competitive metabolite-antimetabolite relationship, as do sulfate and selenate. The few experiments that have been conducted with selenomethionine to see whether such relationships exist have met the criteria for competition and have helped to substantiate this hypothesis (85).

One differentiating feature between many organic sulfur and selenium compounds is the greater electronegativity of the selenium atom. In selenopurines and selenopyrimidines (49), and in selenosemicarbazones and related compounds (50), the attraction of electrons by the selenium atom in the selenocarbonyl groups was found to be greater than that of the thiocarbonyl groups. As a result, the carbon-selenium double bond was more highly polarized than the carbon-sulfur double bond, and the selenium compounds were more acidic than their sulfur analogues. The greater biological activity of the selenium compounds in depressing the growth of several fungi, as compared with the activity of the sulfur analogues, was attributed to an increased chelation of copper in the growth medium (51).

Naturally-occurring organic selenium has frequently been used in animal toxicity studies. The exact chemical composition of this organic selenium is still undetermined, but the properties and behavior in isolation procedures used for other amino acids have been thoroughly investigated (64; 89, p. 155). Two groups of selenium compounds can generally be recognized, the organic selenium in species of *Astragalus* and that in cereal grains. In seeds of *Astragalus* the organic selenium is generally more water-soluble than in grains in which it is associated with the proteins. The distribution in vegetative parts of these plants is variable, and depends somewhat on the conditions and stages of growth. The contrasting syndromes that are occasioned when animals consume these plants may be a reflection of the different types of organic selenium compounds. Final identification of these compounds has been in large measure made difficult by their refractory nature.

ENZYME INHIBITION

A number of attempts have been made to correlate the toxicity of various forms of selenium with the inhibition of specific enzymes or enzymatic processes. Since selenite is known to oxidize sulfhydryl groups in vitro, attention has been focused on enzymes which contain these groups. It has been shown repeatedly that the activity of succinic dehydrogenase from various sources is markedly inhibited by selenite as well as by other sulfhydryl-oxidizing agents. More recently it was demonstrated that preincubation of a dog muscle preparation with succinate, fumarate or malonate prevented the action of selenite and that cysteine was able to reverse the inhibition (2, 3).

In another effort to correlate toxicity with enzymatic inhibition, Klug et al. (39) fed rats sodium selenite and found that over a period of days the level of the succinic dehydrogenase in the liver fell. The limitations to such an approach are inherent in the reactivity of selenite with so many possible oxidizable substances before the ion reaches the liver.

Other respiratory enzymes appear to be inhibited by selenite. During anaerobic glycolysis in homogenates of rat liver exposed to nitrogen, increasing concentrations of the ion suppressed CO_2 output, with a maximum inhibition of about 24% of the control at a level of 8×10^{-5} M selenite (23). In contrast to this, yeast cells that had been starved and then supplied with glucose under aerobic conditions showed no

change in CO_2 liberation after exposure to selenite (5). Oxygen uptake under these conditions was depressed (4, 5). With ethyl alcohol as substrate, O_2 uptake by the yeast cells was also inhibited, but not with lactate or acetate, and only 10% with pyruvate (5).

Several other enzymes of the glycolytic pathway have been found to be susceptible to selenite (2). Charcoal treatment of enzyme preparations of dog muscle resulted in a marked increase in sensitivity to the ion of both the triosephosphate dehydrogenase and the isocitrate dehydrogenase. Addition of boiled yeast extract counteracted the inhibition. Codehydrogenase, a yeast extract component which is separated from its enzyme by charcoal treatment, was thought responsible for the antagonism.

Another enzyme strongly inhibited by selenite is pancreas deoxyribonuclease (21). Under experimental conditions the action of this enzyme occurs in two stages, first depolymerization, then hydrolysis of the substrate. Selenite, among other substances tested, inhibited the depolymerization of the nucleic acid. Inasmuch as iodoacetate and iodoacetamide had no effect on the enzyme, the authors concluded that selenite had not inactivated essential sulfhydryl groups but had interacted with essential magnesium ions which are known to form a highly insoluble salt with selenite.

The studies discussed above, and many earlier ones that have not been reviewed here, show that selenite inhibits a number of enzyme systems. Although enzymes from higher plant sources have not been tested, there is little reason to believe that they should respond differently. Since selenite is such a general inhibitor, it is difficult to conceive that the toxicity of this ion to animals and plants is due to inhibition of any one enzyme system. Nor can one always extrapolate from in-vitro enzyme experiments to in-vivo toxicity studies. The observations of Klug et al. (40) are pertinent. These investigators found that a number of organic selenium compounds inhibited preparations of rat liver succinic dehydrogenase more than did selenite, but were no more toxic than selenite when injected into the animal. The authors point out that the lack of apparent correlation between the degree of enzyme inhibition and degree of toxicity "indicates the danger of relying on conclusions based on in-vitro studies of isolated enzymes alone."

There are several other factors, briefly alluded to earlier, that need to be weighed in toxicity studies with selenite. For one thing, the

oxidation by selenite of metabolically important substances could conceivably lower their tissue concentrations to critical levels. This possibility was examined in experiments with rats that were fed selenite or seleniferous wheat for 18 days, during which time the treated animals showed marked weight losses (37, 38). No significant changes in levels of glutathione or ascorbic acid in blood, liver or kidney were observed, even though glutathione had protected rats against injected selenite in earlier experiments (14).

A second factor to be considered in toxicity studies is the effect of the organic selenium compounds that may arise from selenite. The fate of the ion when mixed with organic foodstuffs which contain oxidizable groups is unknown. These treated foodstuffs must of course pass through the many organs of the digestive tract, into the bloodstream, etc. It is conceivable that anywhere along the line, glutathione, cysteine and perhaps other body components react to form organic selenium compounds which are toxic in themselves. The complex that forms between selenite and glutathione was tested and found to inhibit the activity of rat liver succinic dehydrogenase in vitro about four times as strongly as did selenite, and to have about half the LD_{50} (minimum lethal dose) toward rats (40).

Reduction of selenite to elemental, pink selenium by lower and higher plants has been reported many times, but whether organic selenium compounds are formed comparable to those with gluthathione or cysteine remains undetermined.

Although selenate is not reduced as readily as selenite in vitro, there is evidence that its toxicity to a number of systems does involve a prior enzymatic reduction to selenite. The suppression of O_2 uptake by selenate, unlike the action of selenite, is sometimes preceded by a lag period, during which a reduction to selenite has been postulated (89, p. 209). Rosenfeld and Beath (77) examined this possibility and found that selenite could be detected in a number of animal tissue preparations that had been incubated with selenate. Inasmuch as autoclaved tissues could not effect this reduction, it appeared to be enzymatic. There have been several reports that selenate can be reduced to elemental selenium by microorganisms (18, 74), but the higher plants seem unable to carry out this conversion.

Few enzymatic studies with organic selenium compounds have been made. In a series of compounds tested on rat liver succinic dehydro-

genase, the most powerful inhibitor was selenocystine, and in order of decreasing effect were selenohomocystine, β-β'-diselenodiproprionic acid, "selenium tetraglutathione," selenocystathionine, colloidal selenium, sodium selenite and methyl isoselenourea sulfate (40, 42). Selenomethionine was relatively ineffectual. Although the selenocystine was about 13 times as inhibitory as selenite, there was no indication of why this amino acid or any of the others were so toxic.

SUBSTANCES ANTAGONISTIC TO SELENIUM COMPOUNDS

There appear to be two groups of substances, exclusive of a number of materials which do not fall into any definite category, that can reverse the toxicity of selenium compounds. One group consists of sulfur-containing compounds that are effective in plants and microorganisms, but less so in animals. Substances which contain arsenic fall into the second group. They have received more attention recently, particularly because they have proved to be useful antidotes in animals.

SULFUR ANTAGONISTS. In the first group of antagonists, sulfate is the one substance which consistently has been found to counteract selenate toxicity to microorganisms and higher plants. The antagonism is competitive and is best explained on the basis of the structural similarity of the two ions. In biological systems the criterion for a competitive type of antagonism requires that the degree of inhibition be a function of the metabolite/antimetabolite ratio. This ratio-dependence has been reported in a number of growth studies with *Chlorella vulgaris* (84), *Saccharomyces cerevisiae* (19), *Aspergillus niger* (97) and *Desulphovibrio desulphuricans* (74). These results corroborate and extend the earlier experiments that were conducted with a number of higher plants (89, p .148). The competitive relationship also existed in manometric studies with *Desulphovibrio* (73, 74, 75) during H_2 uptake in the reduction of sulfate. Here, sulfate reduction is an energy-yielding reaction for the organism and is quantitatively much greater than sulfate utilization for amino acid synthesis.

The view that sulfate and selenate compete for the enzyme system responsible for the metabolism of sulfate gathers support from recent work with sulfate "activating" enzyme systems. One such system, prepared from animal and *Neurospora* tissues by Hilz and Lipmann (28), formed an "active" sulfate from ATP and sulfate and was checked by selenate, although under the experimental conditions sulfate was

unable to reverse this inhibition. Wilson and Bandurski (99) discovered a similar reaction in yeast, and their data suggested the formation of an "active" selenate. The purified sulfurylase, which yielded stoichiometric amounts of adenosine phosphosulfate and pyrophosphate according to the following equation:

$$\text{ATP} + \text{Sulfate} \longrightarrow \text{AMP—Sulfate} + \text{Pyrophosphate}$$

was reported to have given only trace amounts of adenosine phosphoselenate (1). Experiments to show actual competition between sulfate and selenate in these systems were not reported. The importance of research of this nature and its bearing on sulfur metabolism and selenate activity cannot be overstressed.

In none of the studies with selenate, when substances other than sulfate were tested for antagonism, has a competitive relationship ever been observed. Instead, a number of sulfur-containing metabolites, such as sulfite (73, 74), cysteine (18, 20, 97), homocystine (97), methionine (17, 20, 84, 97), thiamine (20) and glutathione (18, 97), have been reported to counteract selenate toxicity in a non-competitive manner. The simplest interpretation that most investigators have made is that the enzyme system which reduces sulfate is checked by selenate, consistent with the Michaelis concept of interference by structural analogues. Cells which are impeded at any one enzymatic step may be relieved if they are supplied with products whose formation depends on the normal functioning of the inhibited enzyme. The non-competitive antagonism exhibited by various sulfur-metabolites against selenate is in accord with this concept.

The action of sulfur metabolites toward selenite, on the other hand, appears to differ from that toward selenate and supports the contention that the two ions exert their toxic effects in plants via different mechanisms. Sulfate was tested but could counteract selenite toxicity to several of the higher plants only at very low selenium levels (89, p. 149). Nor could this ion improve the growth of *Escherichia coli* nor restore the ability of the cells to utilize glucose (63). Among the organic sulfur compounds, cystine, one of several other substances used, offered little protection to corn plants (89, p. 144). Cysteine, however, was reported to have allowed *E. coli* to metabolize glucose in the presence of selenite without concomitant growth (63). The data of these latter experiments do not permit any distinction between a metabolic antagonism by cysteine and a non-enzymatic reaction with selenite.

Such a reaction, conceivably, might decrease the concentration of the ion to levels permitting certain metabolic processes to occur in the absence of growth.

Although plants and microorganisms inhibited by selenate respond to organic sulfur metabolites, the use of these substances to counteract the toxicity of selenium compounds in animals has not been effectual. High protein levels are known to decrease the toxicity of seleniferous diets in rats, but the reports on the results obtained with methionine are conflicting. In two recent papers, methionine again failed to protect rats that had been fed seleniferous wheat (34, 35). On the other hand, methionine, as well as choline and betaine, which are also known to be methyl donors, offered partial protection to rats against selenite (53).

The assumption often has been made that selenate, unlike most antimetabolites, can be converted by plants into organic forms by the same enzymes that convert sulfate. These organic selenium compounds in turn have been thought to be competitive. This was the case with DL-selenomethionine and L-methionine, which constituted a pair of competitive antagonists as judged by the growth responses of *Chlorella vulgaris* (85). The selenium analogue also reduced the incorporation of DL-S^{35}-methionine into the proteins of actively growing cells of a methionine-requiring mutant of *E. coli* (11, 12). Antagonism has likewise been demonstrated between selenocystine and cystine. The in-vitro uptake of radioactive L-cystine by human leukemic leukocytes was decreased by prior incubation of the cells with either D- or L-selenocystine (94); the racemic mixture acted similarly on Murphy lymphosarcoma tumor cells of the rat (95). In leukemic patients, oral administration of the selenium analogue resulted in a lower uptake of the S^{35} L-cystine by the isolated leukocytes (96). Criteria to establish the competitive nature of the observed antagonisms were not applied, but it is likely that the sulfur amino acid would also prevent uptake of its selenium analogue. Whether antagonism between these two compounds exists in plants and microorganisms is an open question.

ARSENIC ANTAGONISTS.- Among the arsenic-containing compounds which offer partial or complete protection against selenite poisoning in animals are arsenite, arsanilic acid, 3-nitro, 4-hydroxyphenylarsonic acid, triphenyl-arsine, para-hydroxyphenylarsonic acid, arsenomethane arsenic-1, 2 disulfide, and dodecylamine p-chlorophenylarsonate (24, 25, 46, 61, 62, 92, 93). Several attempts to correlate the antagonism of

arsenic compounds with the metabolism of selenium in the animal body have shown that no significant changes occurred in absorption, excretion or tissue deposition when selenite or seleniferous corn were administered to rats that were simultaneously fed arsenite or arsenate (36, 69). On the other hand, the expiration of volatile selenium compounds was reduced by the injection of sodium arsenite (33). In the livers of rats fed selenite and arsenite, succinic dehydrogenase activity, after an initial drop, rose to higher levels than in rats fed selenite alone (39).

Yeast respiration also has been used as a test system for arsenic effects. In many such studies, phosphate buffers are used, and under these conditions, Berry and Pengra (4) were unable to demonstrate antagonism between arsenite and selenite. Nor was antagonism evident in growth studies with yeast (66) and *E. coli* (63). In the absence of phosphate, however, selenite inhibited the O_2 uptake of yeast to a greater extent, indicative of an antagonism by phosphate (4). This relationship was studied more extensively by Bonhorst (5) who was able to reverse selenite inhibition of yeast respiration with phosphate as well as with arsenite or arsenate. A combination of phosphate and arsenate was effective, too. The relationships between selenate and these ions also were checked and found to differ, since selenate inhibition was not influenced by phosphate but did respond to a combination of phosphate and arsenate.

These antagonisms have yet to be explained. Arsenate is known to prevent the phosphorylation of 3-phosphoglyceraldehyde, one of the glycolytic steps essential for the synthesis of ATP, but does not inhibit CO_2 production. Bornhorst suggests that this reaction may be a possible site at which selenite, arsenate and phosphate interact. Although arsenite is known to inhibit the oxidative decarboxylation of α-keto acids, its antagonism to selenite interference with respiration is still unclear.

OTHER ANTAGONISTS. The amino acid histidine has been reported to allow cells of *E. coli* to metabolize glucose in the presence of selenite, although growth was completely inhibited (63). Among a number of other substances that have been tested as possible antidotes to animal poisoning by selenium compounds, and that have given some protection, are linseed oil meal and several of its fractions (22, 61, 62, 71, 80) as well as ACTH (70).

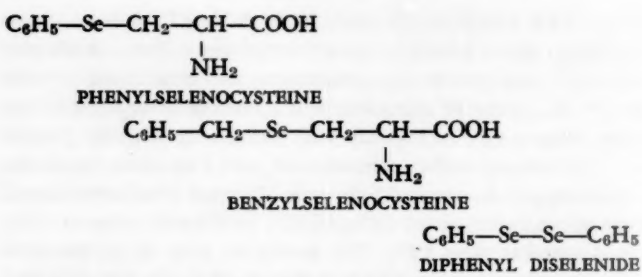
ABSORPTION OF SELENIUM COMPOUNDS

The competitive relationship that exists between sulfate and selenate, as evidenced from growth experiments, has also been found to exist during the absorption of these ions into a number of organisms. The selenium content of plants grown with selenate is inversely proportional to the external sulfate concentration, and, even more important, it is a function of the external S/Se ratio. This has been demonstrated in many of the higher plants (89, p. 142), in *Chlorella vulgaris* (84) and in *Aspergillus niger* (97). The absorption rates of the two ions by barley roots have been studied with the use of tracers (16, 45), and the kinetic data, when analyzed mathematically by the Lineweaver-Burk method for enzymes, were compatible with the view that the ions competed for a carrier at the cell membrane.

Competitive antagonism between L-methionine and DL-selenomethionine also existed during the absorption process in *Chlorella* (85). The selenium contents of the cells were inversely proportional to the external concentrations of the sulfur amino acid, and at a constant S/Se ratio the cells contained the same concentrations of selenium.

These facts indicate that selenate exerts its toxic action in these organisms, at least in part, by diminishing the rate at which sulfate is absorbed, and that selenomethionine interferes with the absorption of L-methionine. In a sense, the cells can be considered as growing under a series of sulfur starvation levels which depend on the external concentration of the seleno-analogue. However, in a methionine-requiring mutant of *E. coli,* even though selenomethionine antagonized the incorporation of S^{35}-methionine into the cell proteins, the seleno-amino acid was apparently utilized for growth in place of methionine (11, 12).

As discussed earlier, selenocystine antagonized the in-vitro uptake of radioactive cystine by human leukemic leukocytes (94) and by Murphy lymphosarcoma tumor cells of the rat (95). Two other structural analogues of cysteine — phenylselenocysteine and benzylselenocysteine—were effective in the leukocytes (94), but the benzyl derivative increased, instead of decreasing, the in-vitro uptake by the tumor cells (95). Selenium compounds, such as diphenyl diselenide, selenium oxide and selenic acid which had no structural relationship to cystine, were ineffective in the leukemic cells (94). In-vivo studies with rats that were fed selenocystine and then injected with cystine showed a lowering of the radioactivity that had reached the tumor



PHENYLSELENOCYSTEINE

BENZYLSELENOCYSTEINE

DIPHENYL DISELENIDE

cells but a marked increase in activity of the liver cells (95). In-vitro experiments with liver cells. to check this relationship, were not reported.

METABOLIC REQUIREMENTS FOR SELENIUM COMPOUNDS

In spite of the evidence that selenium compounds are toxic to many organisms and inhibit a variety of enzyme systems, there are several instances in which this element has been reported to play an essential metabolic role. A number of species of *Astragalus* are always associated with seleniferous soils, and absorb and convert to organic forms relatively large amounts of selenium. Trelease and Trelease (90, 91) have grown several of these species under controlled greenhouse conditions and have demonstrated that the incorporation of selenite into the culture medium markedly stimulated the growth of these plants. This was probably the first work that indicated an essential role for the element.

Several laboratories have recently demonstrated that experimentally-induced vitamin E deficiency symptoms in some animals can be prevented by a number of selenium-containing materials (15, 65, 81, 82, 83, 88). The necrotic degeneration of divers organs of the rat that results when vitamin E is removed from the diet was prevented by Factor 3, a substance isolated from natural foodstuffs, and which was shown to contain organically-bound selenium of undetermined nature (82). Sodium selenite and, to a lesser extent, potassium selenate were also effective. In chicks, exudative diathesis, which ensues from lack of the vitamin, will not develop if the experimental diet is supplemented with Factor 3 (81), vitamin E-free casein (65, 81), or any one of a number of defatted animal tissues (65). Analysis of defatted kidney, one of the effective tissues, disclosed the presence of small

amounts of selenium (65). The chick symptoms also responded to selenite (65, 81); to a crystalline substance (81) isolated from plant material and tentatively identified as a mixture of selenocystathionine and its sulfur analogue (29); and to elemental selenium which, however, was somewhat less effective (81). Vitamin E-deficient pigs also responded to selenite (15).

The precise metabolic function of selenium in the findings described above is unknown. It has been suggested that selenium may be an essential trace element because of the exceedingly small amounts that are effective in the vitamin E-deficiencies (less than one per cent of the previously established chronic toxic dose) and the apparent universal presence of the element in animal tissues (81). On the other hand, it has been pointed out that, since vitamin E (alpha-tocopherol) itself will prevent the deficiencies, the interchangeability may indicate that there is no need for selenium in the presence of adequate vitamin E (88).

The one reference to a specific enzyme requirement for selenite is by Pinsent (72). The development of a high formic dehydrogenase activity in *E. coli* and other members of the coli-aerogenes group required the presence of selenite, molybdate and ferrous iron. In this study, salts were purified by rigorous methods and special precautions were taken to avoid contamination of the chemicals and media. The enzyme apparently was adaptive in nature, for in the absence of selenite and molybdate no enzyme activity could be detected, even though growth was normal. In manometric studies of cells grown without the two ions, addition of selenite and molybdate gave no increase in oxygen uptake in the presence of formate as substrate. Selenate, tellurite and tellurate could not replace selenite, and molybdate could not be replaced by vanadate, chromate, tungstate or uranyl ion. According to the author's calculations, full enzyme activity developed when the medium contained 5000 molybdate ions and 5000 to 10,000 selenite ions per cell. These small amounts ruled out the possibility that impurities might have been directly responsible. A mechanism for these interesting specific effects is not known.

A slight stimulation of recrystallized pig heart fumarase, the enzyme which catalyzes the hydration of fumaric to L-malic acid, is reported for selenate and sulfate (48). Other ions, e.g., arsenate and phosphate, however, were far more stimulatory.

Another instance of a selenium requirement involves the oxidation of elemental selenium to selenic acid by a rod-shaped bacterium isolated from soil enriched with the element (47). This autotroph apparently derived its energy from the oxidation in a manner comparable to the action of *Thiobacillus thiooxidans* on sulfur. Additional details have not been published (Waksman, personal communication).

EFFECTS ON DEVELOPMENT AND MORPHOGENESIS

Profound changes in development and morphogenesis can be induced by exposure to selenite. The filamentous growth of a *Candida albicans* mutant and of other filamentous fungi was transformed into a budding, single-celled, yeast-like growth by incorporation of the ion into a medium which contained yeast extract (60). Because the degree of inhibition was no greater than in the normal yeast-like *Candida*, the conclusion was drawn that the selenite had promoted division but had not retarded the filamentous type growth. An opposite effect, inhibition of cell division, was observed in hanging drop cultures of chick frontal bone cells exposed to sublethal doses of selenite (30). Cell division at metaphase and subsequent nuclear reconstruction were partially inhibited, but entry of the cells into prophase was not prevented. That selenite might have some affinity for the nuclear apparatus is indicated by the development of dark, refractile patches in cells of *Corynebacterium diphtheriae, C. fasciens* and *Bacillus cereus* when grown with selenite or tellurite (26, 27). These observations were attributed to a localized deposition of selenium (or tellurium), since the patches appeared to be in defined regions which were suggested as sites of nuclear structures. The extent to which these few limited observations on cell division can be correlated is uncertain.

At a higher level of morphogenesis, the embryonic development of the sand dollar *(Dendraster excentricus)* has been followed in the presence of selenite (79). Continuous exposure to the ion caused extensive changes, especially along the polar axis. Bilateral symmetry was partially or completely eliminated, and other distinct structural alterations occurred at different stages of the developing embryo. These deformities are reminiscent of the variations in the developing chick embryo that result from injection of selenite into the air cells of eggs (89, p. 211).

Rat fertility, another aspect of animal reproduction, can be influenced

by selenite (78). When supplied through the drinking water at a level of 7.5 ppm selenium, selenite prevented reproduction in the females but had no effect on the fertility of the males. A level of 2.5 ppm selenium, although not inducing this differential effect, nevertheless reduced the number of young reared by the second generation of mothers.

In view of the reactivity of selenite, and on the basis of the available evidence, it is difficult to attribute any of the results obtained in the preceding papers to a specific effect by this ion. Non-enzymatic oxidation-reduction reactions with essential substances in the external medium or within the cells may have been responsible, or other selenium compounds, formed during these reactions, may have contributed to the observed effects.

A morphogenetic effect, more specific in nature, has been observed with selenomethionine (85). In cultures of *Chlorella vulgaris*, grown with sulfate as the only sulfur source, this analogue was able to uncouple growth in size and weight from cell division, with the consequent development of giant cells. Addition of L-methionine was able to restore cell division. Although the exact mechanism is unknown, these data point to methionine as essential for cell division.

The importance of cystine in the metabolism and proliferation of malignant cells, and the antagonism of selenocystine toward the uptake of the sulfur-containing amino acid by such cells, have been used by Weisberger and co-workers as a basis for the treatment of tumor growth in rats (95) and for retarding the proliferation of leukocytes in human leukemia (96). The injection of selenocystine into rats implanted with Murphy lymphosarcoma cells caused a marked reduction in the average tumor area, although some of the 36 treated animals were unaffected. In addition, the number of tumor regressions was increased as a result of this therapy. Benzylselenocysteine, which had had no effect on the uptake of cystine, was also ineffective on the growth and regression of the tumors. In four human leukemia patients, oral administration of selenocystine caused a rapid drop in leukocyte count, whereas diphenyl diselenide did not, in correlation with the results of the in-vitro absorption experiments. Clinical observations revealed, among other things, a disappearance of the immature leukocytes from the bloodstream and a decrease in the abnormal size of the spleen, one of the centers of leukocyte formation. Unfortunately, severe

uncomfortable side effects prevented continuation of the therapy over prolonged periods of time.

The precise mode of action of selenocystine in preventing the growth and development of tumors and the proliferation of leukocytes is unknown. The authors suggest, as one possible mechanism, an inhibition of succinic dehydrogenase as has been observed in other organisms (40). On the other hand, the close correlation between the effects of selenocystine on the uptake of cystine by malignant cells, and on their growth and development, strongly suggests that the selenium analogue interfered with the metabolism of cystine by these cells.

BIOSYNTHESIS OF ORGANIC SELENIUM COMPOUNDS

The ability to differentiate between synthesis along enzymatic pathways and synthesis by non-enzymatic mechanisms is the crux of the difficult question of the origin of selenium-containing proteins. In organisms which possess metabolic pathways for the utilization of sulfate, the evidence that may be cited for an enzymatic conversion of selenate includes, among other features, the structural similarity between the two ions, their competitive relationship, the enzymatic formation of an "active" selenate, and the competition between L-methionine and DL-selenomethionine. In animal systems, where sulfate cannot satisfy the sulfur requirements for protein synthesis, it is known that selenate is reduced to selenite and that the selenium of either ion can become bound to proteins. An answer to how the selenium becomes incorporated into animal proteins must take cognizance of the fact that selenite can participate in non-enzymatic oxidation-reduction reactions under physiological conditions with known metabolites and thereby form organic selenium complexes.

No one as yet has demonstrated unequivocally the biosynthesis of selenium analogues of sulfur metabolites. Although Horn and Jones (29) did isolate from soil-grown *Astragalus pectinatus* a crystalline material which they tentatively identified as an isomorphic mixture of the selenium and sulfur analogues of cystathionine, the components of this mixture were never resolved.

In an attempt to clarify this problem, Smith (86) undertook the analysis of seleniferous wheat, corn and *Astragalus bisulcatus* by paper chromatography. Strips of the chromatograms were cut out and analyzed for selenium. Most of the selenium from the wheat and corn seed pro-

tein hydrolysates was localized in regions that corresponded to the positions of synthetic selenocystine and selenomethionine, with some unknown selenium compounds in other areas. The selenium in several preparations of the *Astragalus* could not be identified as closely. The location of the selenium on chromatograms of aqueous seed extracts differed from that of the synthetic seleno-amino acids; and the chromatograms of aqueous extracts of the plant tops showed a rather general distribution over the entire paper. Acid hydrolysis of the *Astragalus* seed proteins apparently resulted in decomposition of the organic selenium, since the filtered hydrolysates were free of selenium. These differences between *Astragalus* and the grains are in keeping with the previously known chemical properties of the organic selenium in the two groups of plants.

In contrast to these data are the results of Johnson and Whitehead (31, 32) and of Whitehead et al. (98) who applied column chromatography to several protein hydrolysates of wheat plants that had been grown with radioisotopes of sulfate, selenate and selenite. They were unable to detect any radioactive selenium-containing amino acids in either the cytoplasmic proteins of the leaf or seed proteins. They did find, however, that the radioactivity of the protein hydrolysates was associated with column eluates that gave positive ninhydrin tests and which, in the leaf cytoplasmic protein fraction, contained a peptide of undetermined nature. The decomposition of synthetic selenocystine in boiling acid solutions that are used for protein hydrolysis could account for the failure to detect this amino acid. The absence of radioactive selenomethionine from the protein hydrolysates and the known stability of this amino acid under conditions of protein hydrolysis lead to the conclusion that it had not been synthesized, under the conditions of the experiments, by the wheat plants. Whether the results were secured with the wheat that had been grown with either the selenate or the selenite was not indicated. The form of selenium given might have a bearing on the nature of the organic selenium compounds isolated from the plants.

Another instance of selenium in proteins was reported by Nickerson et al. (60) who detected the element in the proteins of isolated cell walls from a strain of *Candida albicans* grown in the presence of selenite. Further details were not given.

In work initiated primarily to see whether variations in protein structure could be induced by environmental changes, Cohen and

Cowie (11) and Cowie and Cohen (12) discovered that selenomethionine supported the growth of a methionine-requiring mutant of *E. coli* in the absence of the sulfur amino acid, provided that sulfate was present to supply the non-methionine sulfur requirements. The mutant grew at a reduced exponential rate in the presence of the seleno-analogue during a number of subcultures that corresponded to more than 100 generations. The possibility that such growth was due to a reversion to the methionine-requiring wild type was checked, but this had not occurred. Nor could radioactive methionine be detected in the proteins of the cells given labeled sulfate. Cystine was highly radioactive, however, as expected.

Competition appeared to exist, since the selenomethionine reduced the rate of cellular incorporation of S^{35}-methionine. These results strongly suggest that the growing cells had used selenomethionine in place of the sulfur amino acid. Assays for the enzyme β-galactosidase gave values that amounted to about one-third that of the control cells and showed that the cells had been able to synthesize active proteins in the presence of the unnatural analogue.

In order to determine whether forms of selenium other than selenomethionine could replace sulfur, sub-inhibitory levels of radioactive selenite were supplied to the wild type and to mutant *E. coli* cells under a variety of experimental conditions. Wild type cells, upon transfer to a sulfur-deficient medium, exhibited a two-phase growth curve. It had previously been established (76, p. 330) that during the first, more rapid phase, the cells consumed their internal store of glutathione as well as the residual sulfur in the medium, and that during the second, slower phase, when alcohol-soluble proteins were utilized for growth, the addition of sulfate initiated normal exponential growth. Since addition of selenite produced no effect, it was concluded that selenium in this form was unable to replace sulfur in *E. coli* under these conditions.

Inasmuch as selenomethionine had replaced methionine, other conditions were sought to see whether selenite could serve for the synthesis of selenoglutathione or selenocysteine. In these experiments, methionine was routinely added to the medium used for the wild type cells in order to exclude any influence of selenite on methionine synthesis from sulfate (12).

A two-phase growth curve, similar to that of the wild type, resulted

when the mutant was transferred to a medium which lacked sulfate but which included methionine. Although selenite again had no effect on growth, it was discovered that the radioactive selenium, nevertheless, had become incorporated into the residual proteins at a rate directly proportional to the new cell growth during both phases.

Hydrolysis and chromatography of the residual proteins of the mutant cells revealed that the radioactivity was localized in the region where cysteine occurred, but a direct comparison with selenocysteine, which was unavailable, was not possible. This evidence suggested that selenocysteine could not be synthesized from selenite alone, but that in the presence of limiting amounts of sulfate or internal glutathione the cells were able to produce a selenium-containing material that chromatogrammed as did cysteine. Under these conditions the cell fractions in which glutathione normally appeared showed no radioactivity, an indication that selenoglutathione had not yet been synthesized.

An experiment to learn whether competition between sulfate and selenite existed during protein synthesis in the wild type demonstrated that the two ions had reduced the incorporation of one another.

From their results the authors have inferred that selenite was metabolized and incorporated as selenocysteine into proteins when the *E. coli* cells were supplied with limiting amounts of sulfate or contained internal sources of glutathione (12).

As first glance selenite seems to resemble sulfite, which has been implicated as an intermediate in the enzymatic reduction of sulfate in microorganisms and higher plants. On this basis one could suppose that selenite would replace sulfite in the enzymatic scheme. However, as was discussed earlier, the two ions differ markedly in chemical properties, selenite being an oxidant and sulfite a reductant. The question arises whether the combination of sub-inhibitory levels of selenium with the residual proteins of *E. coli* at a rate proportional to cell growth may have been due to a non-enzymatic complex formation with the sulfhydryl groups of newly synthesized proteins. The failure to detect selenoglutathione would be in line with this reasoning. Selenodiglutathione, which results from the non-enzymatic reaction between selenite and reduced glutathione (67), might be expected to appear if sufficient

reduced glutathione were available within the cells and if the complex behaved as does glutathione in the fractionation procedures that were used.

The kinetic experiments in which sulfate and selenite reduced each other's incorporation into the residual cell proteins would argue in favor of an enzymatic conversion, since sulfite is also able to prevent sulfate uptake (76, p. 335). The argument would have been strengthened had the wild type cells been grown in the absence of methionine to permit synthesis of the amino acid from sulfate. If selenite were converted enzymatically, radioactive selenomethionine could have been expected on the chromatograms.

The detection of radioactivity in an area of the chromatogram where cysteine occurs cannot be taken as final proof of the presence of selenocysteine. The work of Klug and Petersen (41), discussed earlier, is especially important in this respect, for their results illustrated that the selenium-dicysteine complex, formed upon reaction of selenite with cysteine, chromatogrammed very close to cysteine when a phenol solvent was used. The dearth of chemical information with regard to the reaction products between selenite and sulfhydryl or other tissue components makes difficult the positive identification of radioactive spots on chromatograms prepared from tissues of organisms that have been exposed to labeled selenite. For this reason, a critical proof to substantiate either a metabolic, enzymatic incorporation of selenite into proteins, or a non-enzymatic association, requires further basic chromatographic work and/or the isolation and chemical characterization of the radioactive selenium compounds.

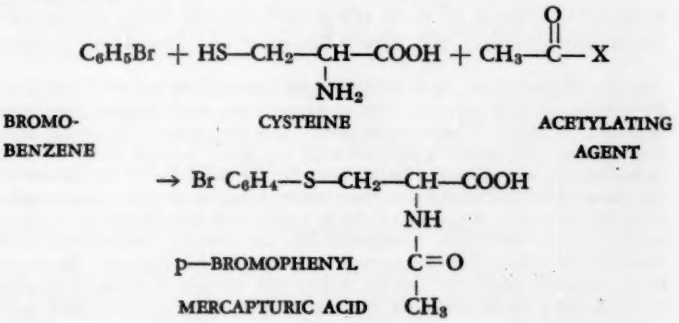
In animals the injection of inorganic selenium salts results in the combination of the selenium with a number of tissue proteins (89, p. 201). The character of this binding has been the subject of a series of tracer studies by McConnell and co-workers. They established that traces of selenate selenium[3] became combined in the rat with several of the milk proteins (52), and in the dog with several of the serum proteins and with both the hemin and globin moieties of hemoglobin (54). Fractionation of the dog bile disclosed the presence of selenium in the bile pigments and in bilirubin, a metabolic decomposition

[3] In animals, selenate is reduced to selenite, which presumably is the reactive ion.

product of hemoglobin (55). Injection of $SeCl_4$[4] also resulted in the combination of the selenium with dog serum proteins (58).

Dialysis of the serum proteins at several pH values (54) and of the biliary selenium (55) revealed that much of the selenium was non-dialyzable, an indication that the selenium had not been adsorbed on the surface of the protein but was probably in an organo-selenium complex. To ascertain the nature of this complex, radioactive dog liver proteins were purified and hydrolyzed, and the constituent amino acids studied by ion exchange, precipitation techniques and paper chromatography (59). The radioactivity in the hydrolysates behaved like amino acids in these studies, and was localized in three spots on the chromatograms. The highest activity was located in the areas of synthetic cystine and selenocystine. The second highest concentration occurred near methionine and selenomethionine, and the least active spot was adjacent to the leucines. In none of the chromatograms, however, did the activity coincide exactly with the positions of the known amino acids, possibly, as suggested by the authors, owing to the large quantities of hydrolysates used in order to get sufficiently high activities. The locations of these spots corresponded to those found by Smith for the selenium in hydrolysates of seleniferous wheat and corn proteins (86).

Other work that suggests a synthesis of selenocysteine involves the treatment of selenized animals with bromobenzene which causes urinary excretion of selenium (89, p. 185, p. 207). Administration of bromobenzene to healthy animals results in the excretion of p-bromophenylmercapturic acid which contains a cysteine moiety:



[4] $SeCl_4$ hydrolyzes to H_2SeO_3 in aqueous medium.

McConnell and co-workers (personal communication) have analyzed the mercapturic acid urine fraction of animals injected with radioactive H_2SeO_3. Of the total urinary Se^{75}, 8-15% was extracted with chloroform, and 2.1-3.5% of the activity in the solvent was in the crystalline mercapturic acid. Paper chromatography was used to confirm these results. The investigators interpreted their data to indicate that the selenium had formed a compound analagous to mercapturic acid.

The data presented by McConnell and co-workers are strong presumptive evidence for the existence of the two seleno-amino acid analogues and possibly others in the animal proteins. Nevertheless, since selenite reacts readily with tissue constituents to form organic complexes not yet fully characterized, but which possess some of the properties of amino acids, including positions on paper chromatograms close to those of the sulfur amino acids, it is difficult to assess these results with finality.

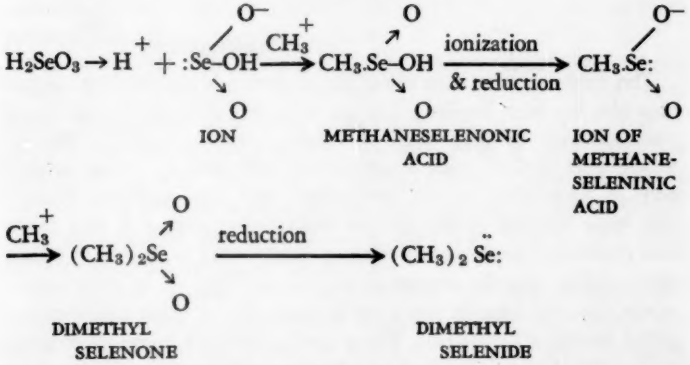
Should these compounds actually prove to be selenocysteine and selenomethionine, the question that arises is whether a mechanism exists in animals for the further reduction of selenite.[1] McConnell and Wabnitz (59) suggest that the enzymatic incorporation of sulfide into cysteine, which has been established by tracer techniques, may have its counterpart in the metabolism of selenium. This would require a reduction of selenite to the level of selenide, a transformation which, as discussed below, can occur, since dimethyl selenide, $(CH_3)_2Se$, has been detected in animals after the injection of selenate.

The methylation of inorganic selenium compounds, a subject reviewed by Challenger (8, 9), is a well-established instance of a biosynthesis of organic selenium compounds. Certain fungi, among them *Scopulariopsis brevicaulis, Aspergillus niger* and *Penicillium notatum,*

[1] At the completion of this review, a paper appeared by Dziewiatkowski and DiFerrante (14a) bearing on this problem. They reported that after intraperitoneal injection of radioactive sulfate into rats, about 5% of the radioactivity that had become associated with the serum proteins could be found as methionine and cystine. A mechanism for the reduction of the sulfate was not discussed by the authors, but these results bring up an interesting question. Was the conversion of the sulfate due to a metabolic transformation by the rat tissues, or was the sulfate transported into the intestine, metabolized by the intestinal flora, and then transported back into the bloodstream? An answer to this question might also help to explain the presence of traces of selenium in the amino acids isolated from the liver proteins of rats injected with radioactive selenate (59).

are able to convert Na_2SeO_4 or Na_2SeO_3 to dimethyl selenide. This compound also has been isolated from rat exhalations after the animals had been injected with radioactive selenate (56).[6] Upon injection with selenite, volatile unidentified selenium compounds have been detected (33, 68).

According to Challenger and co-workers, methylation by fungi proceeds through a succession of methylating and reducing steps. The following mechanism has been suggested (8):

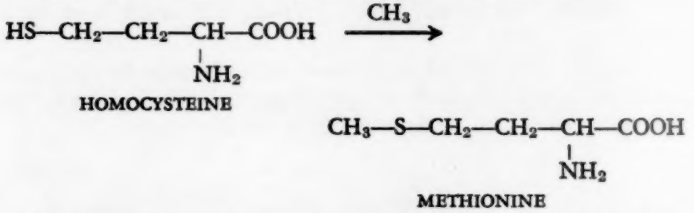


These equations are based primarily on the utilization of the methyl, ethyl and n-propyl seleninic acids ($R\text{-}SeO_2H$) by some of the fungi to yield the corresponding dimethyl, methyl ethyl, and methyl n-propyl selenides ($R\text{-}SeCH_3$). The postulated intermediates, however, have not been isolated.

The question of the origin of the methyl groups has been resolved by the use of compounds with labeled methyl groups (10, 13). Methionine proved to be the best methyl donor, whereas the C^{14} of the methyl groups of choline or betaine barely appeared in the dimethyl selenide. The possibility that formate might serve as a source of the methyl carbon was tested, but the C^{14} in the selenide was also low. These data demonstrated that transmethylation occurred from methionine to the selenite ion. This view was strengthened by the finding that homo-

[6] The ability to methylate selenate has been suggested as a possible detoxification mechanism in animals, since mice and rats were able to withstand doses of injected dimethyl selenide which were many times the toxic doses of the inorganic ions (57).

cystine, the oxidized form of homocysteine which is a precursor of methionine, when present in addition to labeled betaine or formate, increased the amount of radioactivity in the dimethyl selenide:



HOMOCYSTEINE

METHIONINE

The methylation step in which homocysteine is converted to methionine also has been implicated in the reduction of selenite to elemental pink selenium by the action of *Neurospora crassa* (102). Mutants which were blocked after homocysteine were able to reduce selenite only in proportion to the methionine that was supplied. Mutants that were blocked earlier in the biochemical sequence, and therefore could methylate homocysteine to methionine, were able to reduce more selenite than the amount of methionine supplied. In these experiments, dimethyl selenide could not be detected, nor could this organism utilize methyl seleninic acid. These results indicated to the investigator that selenite was reduced in *Neurospora* by a mechanism differing from that suggested by Challenger (8) but somehow still involving methionine.

CONCLUSIONS

The manner in which selenium is built into protein molecules remains an elusive biochemical problem. In the higher plants and microorganisms which utilize sulfate, the evidence, though indirect, leans toward a competitive antagonism between sulfate and selenate, with the latter being metabolized along the sulfur pathways. The resultant organic selenium analogues have not been conclusively identified, but synthetic selenomethionine appears to function as an antimetabolite. Since selenite does not exhibit typical antimetabolite characteristics, its mode of action in plants seems to be entirely different from that of selenate. Although animals are unable to utilize sulfate for protein synthesis,[7] a system exists for the reduction of selenate to selenite. At this point there seem to be two possible divergent mechanisms

[7] See footnote 5.

whereby the selenium becomes associated with proteins. First, selenite can be reduced in the animal body to the selenide level of dimethyl selenide, and the selenide-selenium may then possibly follow the route of sulfide. Second, selenite is known to undergo non-enzymatic oxidation-reduction transformations in vitro with sulfhydryl-containing substances such as cysteine and reduced glutathione. The resulting organic selenium compounds possess amino acid properties and chromatogram close to the sulfur amino acids, so that positive identification of enzymatically formed organic selenium compounds by the technique of paper chromatography is made difficult.

The integrity and plasticity of protein molecules require the presence of the sulfur amino acids, methionine and cysteine, and of sulfhydryl groups and disulfide bridges. To what extent the sulfur can be replaced by selenium without impairment of the functioning of the proteins has been little explored. That such a replacement may prove possible is evidenced by the ability of a methionine-requiring mutant of *E. coli* to grow exponentially, although at a somewhat reduced rate, with selenomethionine replacing methionine.

A number of studies of enzyme inhibition by selenite have demonstrated that catalysts with readily oxidizable sulfhydryl groups are generally the most susceptible to this ion. But since other enzymes can also be affected, it is difficult to ascribe to selenite a specific enzymic site of action. Similarly, morphogenetic changes or toxic symptoms induced by the ion probably cannot be attributed to one particular reaction.

A metabolic role for selenium, first indicated for several species of *Astragalus*, appears now to have been extended to animals. This will undoubtedly provide a new biological status for the element.

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PLANT-GROWTH RELATIONSHIPS ON SALT-AFFECTED SOILS [1]

H.E. HAYWARD AND L. BERNSTEIN [2]

Numerous reports dealing with plant responses on salt-affected soils have appeared since the earlier review of this subject in this journal by Magistad (143). In the interim, Hayward and Wadleigh (99), Hayward (93) and Grillot (78) have reviewed segments of the literature in this field. The present article proposes to bring together published material from some areas, notably Japan and Russia, which have been somewhat neglected in previous reviews along with the more commonly available publications. Because of language difficulties, the authors have attempted a selective review of Russian and Japanese papers rather than complete coverage.

INTRODUCTION

Salt-Tolerance Lists

Studies of relative salt-tolerance have dealt mainly with those groups of plants which have economic importance. These include forage, fiber, cereal, vegetable, fruit crops, and ornamental and roadside plants used by man to make his environment more pleasant.

In this connection, many species and varieties of plants have been investigated by workers from The Netherlands, North Africa, the United States, the Union of Soviet Socialist Republics, and elsewhere. In The Netherlands, Beeftink (15) has reported chiefly on fruits; van den Berg (16, 17) and van den Berg and Westerhof (18) on cereals, grasses, field and vegetable crops; Dorsman and Wattel (61) and van Dam (57) on vegetables; Rowaan (177) on horticultural crops; and van der Linde and van der Meiden (134) on deciduous trees. Much of this work was stimulated by the inundations of 1953 and previous floods. Abell (1) has reviewed the Dutch literature since 1954 dealing

[1] Contribution from the U. S. Salinity Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, U. S. Department of Agriculture, Riverside, California in cooperation with the 17 Western States and the Territory of Hawaii.

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with salt tolerance and has prepared a statement giving the crops studied and an appraisal of the work reported.

Salt-tolerance lists from North Africa include those of Grillot (78), in which seven categories of domesticated plants are given, ranging from those which are ultra-sensitive to salt to those which are ultra-resistant; and those of Simmoneau (184) which list cereals, forage grasses, industrial plants, vegetables and fruits. The U. S. Salinity Laboratory (203) has investigated the salt tolerance of field, fruit, forage and vegetable crops, listing about 90 species and varieties. Oganesian (154, 155, 156), in the U.S.S.R., has prepared lists showing the relative salt tolerance of perennial grasses, legumes, and fruit and field crops. Other investigators, mentioned in the following sections, have made comparative evaluations of related species or varieties or of certain groups or families of economic plants.

Soil Terminology and Methodology

Magistad (143) prefaced his discussion of plant response with a description and classification of saline and alkali soils. In the present account, no attempt will be made to review the literature on soils. Instead, definitions of terms commonly employed to designate the soil conditions of major interest will be given to clarify the diverse criteria for soil salinity and related conditions used by different authors.

A "saline soil" is one which contains sufficient soluble salts to affect adversely the growth of plants. It is now almost universally recognized that to evaluate the salinity of soil, the moisture-holding capacity as well as the salt content must be taken into account. Dutch (17, 18) and French (153, 216, 217, 218) investigators have preferred to measure both salt and moisture and to express the salt concentration of the soil solution in grams per liter. This practice is most justifiable when the constituent salts occur in relatively constant proportions as from various dilutions of sea water in Holland. The U. S. Salinity Laboratory (203) has recommended determining salinity by measuring the electrical conductivity of the saturation extract of soil (EC_e). This procedure also takes into account the moisture characteristics of the soil and has the advantage that electrical conductivities are closely correlated with osmotic pressures of soil solutions. A good measure of total salinity is obtained without requiring analysis for specific ions which may vary greatly in their proportions from soil to

soil. Unfortunately, some authors still report salt content on a dry soil basis only, and provide no means for estimating the concentration of the soil solutions.

The lower limit for salinity of soils is not too clearly defined. Recent experiments with salt-sensitive crops have suggested a downward revision of this limit from an EC_e of four to two millimhos (203). For comparison with the scale employed by the Dutch and French investigators, this is equivalent to about two grams NaCl/1 at field capacity.

"Sodium" or "sodic soils," also commonly called "alkali soils," are those which contain a high enough percentage of sodium on the exchange complex of the soil to affect plants adversely. Fifteen percent exchangeable sodium has been suggested as the lower limit for sodium soils, based primarily on the development of unfavorable physical characteristics (203). Recent work has shown that some plants are so sensitive to exchangeable sodium, that severe damage occurs at much lower levels of exchangeable sodium when the physical properties of the soil are still favorable for plant growth. As with salinity, therefore, no clearly defined lower limit for sodic soil may be set up without taking into account the crop or crops in question.

A variety of techniques has been employed in assessing the salt or sodic tolerance of plants. Earlier investigators frequently relied upon an analysis for salt in the root zone of field-grown plants or native vegetation. The extreme variability in salinity and exchangeable sodium in the field limits the usefulness of this method. In recent years, however, Dutch investigators have effectively employed such field observation techniques in studying recently inundated polders to derive data on salt tolerance. In culturing plants experimentally for salt-tolerance studies, every known plant-culture technique has been employed. Sand and water cultures have been effective in revealing specific ion effects and in physiological studies in general. Soil cultures have been widely used for salt tolerance data (85, 115, 116, 136, 151, 153, 216) and for studying the interaction of soil moisture tension and salinity on crop growth (205, 209). Artificially salinized field plots have provided much of the salt tolerance data developed at the U. S. Salinity Laboratory (6, 11, 13, 19-23, 33) and elsewhere (5, 46, 74).

Although saline- and sodic-soil conditions as defined are clearly distinguishable, they frequently occur together to produce saline-sodic

soils. In such cases, investigators have not always clearly distinguished between salt and sodium effects, especially when the two factors were highly correlated as in lands inundated with sea water or brackish waters containing fairly constant proportions of sodium to total cations. Some of the effects attributed to salinity in such cases may have been caused by exchangeable sodium.

Some plants are much more sensitive to salinity during germination and early seedling growth than during later stages of development. However, investigators have not followed any consistent procedure in regard to the stage of development at which salt tolerance is assayed. Because salinization of soils in North Africa normally occurs during the later stages of growth, following application of saline well waters to the fields, Yankovitch (216, 217, 218) generally cultured his experimental plants in non-saline soil until the flowering stage before introducing graded amounts of salt in the cultures. His data, therefore, reflect tolerance during maturation only, and in some cases are markedly at variance with findings which refer to a larger portion of the growth cycle. Other investigators have found it convenient to separate germination studies from those on later stages of growth to avoid the complicating influence of partial stands on yields.

Criteria of Salt Tolerance

The manifestation of salt tolerance in a plant may involve climatic, edaphic, genetic, physiological and pathological factors as well as their interaction. Regardless, however, of the factors involved, salt tolerance can be appraised in at least three ways: *a*) the ability of a plant to survive on saline soils; salt tolerance based primarily on this criterion of survival has limited application in irrigation agriculture but is a method of appraisal which has been widely used by plant ecologists; *b*) the absolute yield of a plant on saline soils; this criterion has the greatest agronomic significance; *c*) relative yield on saline soil compared to similar nonsaline soil; this criterion is useful for comparing dissimilar crops whose absolute yields cannot be compared directly. The U. S. Salinity Laboratory (203), in compiling its lists of salt tolerance, has used the third criterion.

In addition, the units of salinity measurement have varied in different studies. In some cases, salt tolerance has been related to the amount of salt in the soil expressed in terms of percentage on a dry soil basis.

Other writers, such as those in The Netherlands, have based their field-plot studies on a salinity value referred to as a "salt index." This is expressed as grams of NaCl per liter of soil-water at a given depth in the profile which is associated with a definite percentage decrement of a normal yield. The relative salt tolerance of crops given by the U. S. Salinity Laboratory (203) is based upon the conductivity of the saturation extract expressed in millimhos per centimeter (EC_e) at which a 50-percent decrement in yield may be expected when compared to yields of the given plant on a non-saline soil under comparable growing conditions.

The Nature of Salinity Effects on Plants

The presence of excessive concentrations of soluble salts in the root zones of plants may affect plant growth in a number of ways. The increased osmotic pressure of saline soil solutions tends to restrict the uptake of water by plant roots (97). All plants are subject to this influence, although sensitivity to this effect varies widely with plant species. Plants also absorb the constituent ions of the saline solution to varying degrees. This may result, in some cases, in toxic accumulations of a particular ion or in decreased absorption of some essential nutrient. Such toxic or nutritional effects cannot generally be conclusively demonstrated by simply relating plant growth to composition. Compositional changes may closely parallel the osmotic effects, rendering difficult the partitioning of total effect into osmotic and specific ion components. The titles of some papers suggest a direct toxic effect of chloride, but carefully controlled experiments reveal that, for most species involved, isosmotic concentrations of sulfate salts may prove equally inhibitory to plant growth.

Specific Ion Effects

Specific toxicity of chloride and sodium has been demonstrated for a number of fruit crops by comparing the effects of isosmotic solutions of different salts (7, 35, 96). For a number of other fruit crops and trees, presumptive evidence suggests the specific toxicity of these ions. These toxic and nutritional effects will be described in the appropriate sections which follow.

Much of the research on plant response to sodium and chloride has been concerned with their use in fertilizers. While the results of such

studies may obviously not indicate the effectiveness of these ions at the much higher concentrations in which they occur in saline soils, it is pertinent to give brief consideration to these experiments if only because they have strongly influenced thinking on the nature of plant responses to chloride and sodium.

Hayward and Wadleigh (99) have discussed the deleterious effect of chloride salts on the quality of tobacco and potatoes, two crops which are reputedly chloride-sensitive. Smith and Nash (186) found that chloride fertilizers depressed starch accumulation in potato tubers. Latzko (127, 128) attributed decreased translocation of carbohydrates from the leaves of chloride-fertilized potatoes to lowered carbohydrase activity. Bernstein, Ayers and Wadleigh (22), however, found that soil salinity induced by chloride hastened starch accumulation in potato tubers, although yields were markedly depressed. These authors suggested that the high osmotic pressure of the saline soil solution counteracted any specific chloride ion effect on starch accumulation in the tubers.

In addition to reported effects of chloride on carbohydrate metabolism, the chloride ion has also been suspected of affecting the mineral nutrition of plants. Gauch and Wadleigh (72) reported that nitrogen assimilation by bean plants was depressed by high concentrations of sodium chloride or calcium chloride in water cultures. Kretschmer, Toth and Bear (122), working in a nutrient-concentration range, found no consistent effect of chloride versus sulfate on the nitrogen content of 11 crops tested. Reifenberg and Rosovsky (171) tested the effect of serial dilutions of a saline well water on the absorption of nutrients by barley seedlings. They reported little or no effect of chloride in concentrations up to 3000 p.p.m. on the absorption of nitrate or phosphate, but increasing the phosphate or nitrate concentration of the medium depressed chloride absorption. These authors also found that increasing concentrations of sodium had no significant effect on potassium absorption, although increasing potassium did depress sodium absorption. Leonard and Toth (132) found a similar effect of potassium on sodium absorption.

Arnold (4) reported no specific toxicity of chloride for tomato when various proportions of nutrient anions were replaced by chloride. Buchner (36) even attributed a major portion of the stimulative effect of sodium chloride on beets to chloride rather than sodium.

Toth and Kretschmer (199), using Cl^{36}, studied the distribution of chloride in a number of plant species. Highest Cl^{36} accumulations in lima beans were in the pulvini, while in tomato these occurred in the stem and petiole of the lowest fruit cluster. Cl^{36} applied directly to the leaves was translocated both upward and downward. In beets more chloride was found in leaves younger than the treated leaf, while in tomato, wheat and rye, more chloride appeared in the older leaves.

In fertilizer studies with sodium, major attention has been given to beneficial influences of sodium on the yields of crops. For some crops, stimulation is apparent only when potassium is deficient, but Lehr (129, 130, 131) reports that sodium increases yields of oats and flax as well as spinach, even when potassium supply is apparently optimum. The stimulative effect of sodium has recently been discussed by various authors in a sodium symposium (204).

In arid regions sodium fertilizers are generally distrusted because of the tendency for sodium to accumulate in such areas, thus producing the unfavorable effects of a sodic soil condition. Some crops are so sensitive to exchangeable sodium that toxic effects develop even before the level of exchangeable sodium becomes high enough to affect adversely the physical condition of the soil. Martin and Bingham (146) found sodium toxicity symptoms in avocado with as little as 4% exchangeable sodium. Citrus (147), peach (148) and beans (26, 30) are also rather sensitive. Most other crops, such as cotton and alfalfa (41), beets and Rhodesgrass (30), tomatoes (198) and grain crops (168), are tolerant enough so that unfavorable physical characteristics of sodic soils may generally be expected to limit growth and yields. Schreiber, Davis and Overstreet (179) reported the growth of radish seedlings to be maximal at 10 to 20% exchangeable sodium, declining sharply at higher levels. In agreement with many authors cited above, Schreiber et al. correlate plant growth with calcium availability in sodic soils. Above 20% exchangeable sodium, decreased elongation of seedlings was related to loss of calcium. Approximately twice the concentration of exchangeable hydrogen or magnesium was required to effect calcium loss from seedlings compared to exchangeable sodium. Specific toxic effects of sodium and chloride on sensitive fruit crops will be discussed in connection with their salt tolerance.

While sodium and chloride appear to be specifically toxic to only a limited number of crops, boron and bicarbonate in excess are gen-

erally toxic, although plants vary somewhat in their tolerance to these ions. Wadleigh and co-workers found beans (207) much more sensitive to bicarbonate than beets (34), and Dallis grass more sensitive than Rhodes grass (73). Porter and Thorne (167) found the bicarbonate ion concentration to be more effective than CO_2 tension in causing chlorosis of beans and tomatoes. The effect of bicarbonate ion is at least in part attributable to iron deficiency. Since iron chlorosis has been reviewed recently by Brown (31), in relation to lime- and bicarbonate-induced chlorosis, no further discussion of this problem will be attempted in this article. Boron tolerance data will be reviewed in connection with the discussion of individual crops.

GERMINATION

Spotty stands and bare spots, indicative of poor germination or poor seedling emergence, are characteristic features of plant growth on salt-affected soils. In part, these effects may reflect a greater sensitivity of the germinating seed to salinity than of later growth stages, the beet being a notable example (12). More often it represents the result of exposure to greater salinity levels during germination than may be encountered in later growth stages. Salt accumulation in the surface layers of soil frequently results from evaporation and capillary rise of saline waters. It is not at all uncommon, therefore, for seed to be exposed to salinity levels appreciably greater than the average value in the top six or 12 inches of soil. Evaporation, by reducing the moisture content of the soil, also tends to increase both the soil moisture tension and the osmotic pressure in the surface layer of soil. Ayers (8) has shown that increased moisture tension intensifies the effect on germination of a given salinity level. Finally, salt accumulation is greatly intensified by furrow irrigation (140, 208). The roots of a well-developed plant may largely avoid the most saline zones of soil on the surface and under the center of a raised bed, and proliferate and utilize the much less saline soil under the furrow. The germinating seed, however, often cannot escape the effects of the most saline conditions. Bernstein and Fireman (24) have determined the salt accumulation patterns in furrow-irrigated saline soils. The leachable-salt content in a loam soil was found to increase seven-fold in the center of a flat bed following one application of water in the furrows. The shape

and height of beds as well as the duration of irrigation influenced the final salt-accumulation values.

Bernstein, MacKenzie and Krantz (25) correlated germination of a number of row crops with salinity level in the plow layer for a variety of planting and irrigation practices. Management practice was far more important than salt tolerance of the species in determining the success of plantings in saline soils. By suitable planting and irrigation practices, the effective salinity adjacent to the seed may be reduced to only a few percent of that originally present in the soil, instead of the seven-fold increase previously cited (24). Heald, Moodie and Leamer (100) improved the stand of sugar beets in saline soil very considerably by applying preemergence irrigations in rills adjacent to the seed row. Preplanting leaching, of course, tends to improve germination. Dunnewald (64) found germination in a leached saline soil was 81 and 92% for sweet clover and alfalfa, respectively, compared to 52 and 18% for the two crops in the original soil before leaching. The sweet clover was less sensitive to salinity than alfalfa.

Although basin or border irrigation can generally be depended upon to reduce salinity in the surface layer of soil and promote germination, many species fail to germinate or emerge satisfactorily after flooding because of poor aeration or the development of a hard crust due to dispersion of the soil (25).

Ayers and Hayward (12) developed a laboratory technique for determining salt tolerance during germination. They found that the first increments of salinity tend to retard germination and that additional increments tend to reduce progressively the final percent germination. Tolerance during germination was poorly correlated with salt tolerance during later development. Corn was more tolerant during germination than sugar beets or alfalfa, yet it is more sensitive to salinity than either of these crops during later growth stages. Barley, var. California Mariout, was exceedingly tolerant during germination. In a later study (9), significant varietal differences in the salt tolerance of barley were demonstrated.

The dominant effect of osmotic pressure on germination of alfalfa seed was demonstrated by Uhvits (201) who found approximately equivalent effects of isosmotic solutions of mannitol and sodium chloride. Specific salt effects were appreciable at higher concentrations of sodium chloride, as evidenced by an increased proportion of deformed

seedlings and lower germination percentages. Gingrich and Russell (76) and Danielson and Russell (58) studied the effects of soil moisture tension and osmotic pressure on root elongation and Rb^{86} absorption by corn seedlings. The greater effect of moisture tension compared to equivalent osmotic pressures on both processes was attributed to a limiting rate of moisture transmission and Rb^{86} diffusion as moisture film thickness decreased in media adjusted to higher moisture tensions. Helmerick and Pfeifer (101), testing two varieties of winter wheat, found that Yogo gave superior germination compared to Cheyenne when either soil moisture or osmotic pressure was the limiting factor.

In an investigation of the salt tolerance of grain and pasture plants in West Australia, Millington, Burvill and Marsh (151) tested germination on soils of graded salinity. In order of decreasing salt tolerance, the ranking of the grains was cereal rye, wheat, barley, oats. Of the pasture species, sea barley *(Hordeum marinum)* and alfalfa were most tolerant, exceeding Wimmera rye *(Lolium rigidum)*, curly rye *(Pholiurus incurvus)*, *Atriplex semibaccata*, mangold and oats. The contrast between salt tolerance during germination and later stages was very marked, halophytic *Atriplex* and mangold being quite sensitive during germination, even more so than the mesophytic alfalfa.

Kapp (114) found the germination of rice was affected at a salt content of 825 pounds per acre, whereas 3300 pounds per acre was required to affect yield when applied to six-week-old plants. Iwaki and Ota (107) report almost normal germination of rice with waters containing up to 0.06% NaCl.

Ota, Ogo and Omori (160) report that wheat absorbs progressively less water and that germination percentage decreases with increasing concentration of either glucose or NaCl. At higher concentrations of NaCl, growth of the seedlings on the salt was poorer than on the sugar solution (cf. Uhvits, 201). Dilutions of sea water were less toxic than NaCl solutions. Cerighilli and Durand (40) found that low concentrations of sodium chloride seem to stimulate the development of rice but that increasing concentrations rapidly become damaging.

In the adaptation of the cotton plant to salinity, Strogonov (191) found that seeds produced on plants grown under saline conditions possessed no increased tolerance to salinity. Moreover, Strogonov (192) reported that the leaching of cotton seed produced on saline soil

greatly improved germination, suggesting that some water-soluble toxic material was present in these seeds.

Kling (115) germinated tomato, cotton and alfalfa on balanced salt solutions of graded salinity. A 0.1 M solution retarded germination and higher concentrations reduced the percent germination. Kahn, Goss and Smith (112) recently reported that osmotic inhibition of the germination of lettuce seed by 0.15 M mannitol may be overcome by treatment of the seed with gibberellin. The range of effectiveness of gibberellin treatment was not further investigated.

Novikoff (153) reported the effect of sodium chloride solutions on the germination of a number of truck crops. Approximately 50% reduction in germination was obtained at concentrations of sodium chloride of 6 gm./1. in fennel and salsify, 8 gm./1. for lettuce, and 18-22 gm./1. for carrot, eggplant, lima beans, melons, tomatoes, onion, radish and Savoy cabbage. The values for the last group of crops appear inordinately high.

Relatively little is known regarding the effects of exchangeable sodium on germination other than that the dispersed condition of sodic soils is generally deleterious for seedling emergence because of poor aeration and crusting. Bernstein and Pearson (26) found that, if a favorable physical condition of the soil was maintained by the use of a soil conditioner, exchangeable-sodium-percentages up to 60% had little or no effect on the germination of beets or beans. The germination percentage of clover, however, was affected at exchangeable-sodium-percentages above 25 and of alfalfa at 50%. At 50 – 60% exchangeable sodium, the germination of clover was reduced by 90% and of alfalfa by 50%. In the absence of a soil conditioner, germination of all crops was more severely impaired by exchangeable sodium than in the conditioner-treated soil.

Indicator Plants and Lower Plant Forms

Although economic plants have received major attention in salt-tolerance studies, some of the recent ecological and physiological investigations on non-cultivated species should be mentioned. The subject of halophytes was reviewed by Uphof (202), and the water relationships of halophytes has been discussed by Adriani (2) and Stocker (190).

The salt tolerance of marsh plants has been found to be of diagnostic

significance for the classification of marsh lands with respect to land use. Penfound and Hathaway (166) list the salinity ranges for the growth of many marsh species. Allan (3) and Hinde (102) emphasize the water-table and salinity factors in determining the occurrence of marsh-land species. Seashore plants have been studied by Biebl (27) who found halophytes more resistant to plasmolysis by sea water than glycophytes. Takada (196, 197) divided strand plants into three groups — euhalophytes, oligohalophytes, non-halophytes — depending on the relationship of osmotic-pressure fluctuations to chloride and sodium contents of the leaves. The euhalophytes were characterized by fluctuations in sodium and chloride which paralleled the diurnal fluctuations in osmotic pressure. Black (28), however, found that chloride accumulation in *Atriplex hastata* was even less than in tomato and much less than in *A. vesicaria*, even though *A. hastata* tolerated up to 0.6 M NaCl, compared to 0.2 M and 0.4 M for tomato and A. *vesicaria*, respectively. Dubovik (63), likewise, concluded that there was no proportionality between ionic composition of plants and the ionic composition of soils of varying salinity on which they grew.

Migahid and Ali (150) found the osmotic pressure of mesophytes, xerophytes and halophytes to be directly related to salinity and inversely related to the soil-moisture level. Soil salinity was the more decisive factor of the two. Fedorovskii (67) found that the wilting coefficient of soils varied with the plant species and that the available moisture in soils decreased with increasing salinity for a number of crop plants tested. With halophytes *(Salicornia, Salsola)*, however, the wilting percentage held constant or even was lowered with increasing salinity.

Iljin's epidermal-strip technique for studying salt effects on halophytes and glycophytes (103) has recently been employed by Stadelmann (189) who found that desert species had a high resistance to salt solutions. Dearborn (59) has applied the fact of differential salt sensitivity of species in a weed-control practice by spraying fields of canning beets with sodium chloride solutions. The more tolerant beet plants were only temporarily set back by a salt spray, whereas ragweed, mustard and some other weeds were effectively eliminated. However, the more salt-tolerant weeds, lamb's quarters (*Chenopodium album*) and purslane *(Portulaca oleracea)*, were also resistant to the salt spray.

Genkel' (75) found that the stability of the protoplast-cell wall connections determined by centrifugation of tissues in a hypotonic sucrose

solution was essentially as low in halophytes as in hemixerophytes, in contrast to a markedly higher stability detected in euxerophytes.

References to the salt tolerance of lower plant forms are relatively few. Burcik (37) compared effects of salinity and varied relative humidity of the atmosphere on the growth of bacteria and yeasts. Many of the bacteria and particularly the yeasts were able to absorb sufficient water from air to permit growth at relative humidities as low as 95 to 90 percent, whereas most higher plants generally fail to grow when osmotic pressures in the substrate are equivalent to relative humidities of only 98 to 99 percent. Deviations in response to osmotic pressure in the substrate compared to equivalent relative humidities in the air were attributed to specific ion or salt effects. Kaliuzhnyi (113) discusses the water relationships of yeast cells and reports that salt added to the medium decreases the total and particularly the free moisture content of yeast cells.

SALT TOLERANCE OF FIELD CROPS

Most of the major field crops have good to medium salt tolerance, and some of them exhibit very high salt tolerance. Among the latter are barley, sugar beet and cotton. Crops which show medium salt tolerance include most of the other cereal crops—wheat, oats, rice, rye. Flax is moderately salt-tolerant, and corn and sorghum are usually considered to be somewhat more sensitive to salt than the other cereals. Field beans are generally regarded as being a salt-sensitive crop.

Ayers et al. (11) found that barley was one of the most salt-tolerant plants and produced grain satisfactorily at EC_e values of 12-14 mmhos., provided salinity during seedling development was in the range of 1-4 mmhos. If the salinity level at germination was as high as 9 mmhos., then salinity of 12-14 mmhos. in the later stages caused a serious decrease in the yield of grain. Vegetative growth and straw yields were much more sensitive to salinity than was grain production. In a later study, Ayers (9) tested 30 varieties of barley in salinized soil cultures and observed that there were some significant differences among varieties with respect to their salt tolerance at the germination stage. For example, California Mariout germinated well with the soil solution at an osmotic pressure of 19 atmospheres ($EC_e = 24.4$ mmhos.), while Arivat was completely inhibited in its germination at 13.7 atmospheres ($EC_e = 18.5$ mmhos.). Van den Berg and Westerhof (18)

examined soils and crops after the inundations of February, 1953, in The Netherlands. Spring barley was the most tolerant of the field crops, followed by oats, sugar beets, potatoes and flax. Winter wheat showed a reasonably good salt tolerance. Marshall (145), working in Canada, stated that barley is the crop most likely to succeed on saline soil, and found a good stand of barley, although somewhat delayed in maturity, in soil containing 1.6 percent of mixed sodium and magnesium sulfates. Wheat was a little less tolerant but survived at 1% salt. These values are high and indicate the possibility of using some saline areas if the soil-moisture conditions are favorable. Doughty and Stalwick (62), also in Canada, noted 0.4 percent of water-soluble salts as the approximate upper limit of fair crop growth for oats and wheat. At a concentration of 1.09% salt, there was a reduction in yield of 33 to 77 percent. Alfalfa was more tolerant than either of the cereals. Pot tests at the Soil Research Laboratory, Swift Current, Saskatchewan, Canada (188), indicated that oats and wheat were about equally salt-tolerant but were not quite as resistant as alfalfa.

Millington, Burville and Marsh (151), working in Australia, report that, of the cereals, the feed barleys have the greatest capacity for both germination and winter growth on saline soils, but suggest that higher seeding rates are desirable. They grew their plants in various dilutions of sea water and in salinized soils ranging from 0.02 to 0.3 percent sodium chloride.

Singh (185) related the greater salt tolerance of barley compared with wheat to the larger number and the smaller size of the stomata and to the smaller epidermal cells of the barley leaf compared to the wheat leaf. Yankovitch (218), in North Africa, noted varietal differences in the salt tolerance of cereals. He observed that the effect of chlorides on cereals was to shorten the vegetative cycle, darken the color, and reduce the length and weight of the straw. Yankovitch allowed plants to develop two to three months (usually up until flowering) on non-saline soil cultures before introducing graded levels of salinity. Twenty percent reductions in yield of oats and wheat were caused by NaCl concentrations of approximately 10 gm./1. of soil moisture. Equivalent yield reductions for sugar beet occurred at 27 gm./1., and for flax at 8 gm./1., indicating the moderate salt tolerance of the grain crops under these experimental conditions. Other studies by Yankovitch (216) indicated oats to be more salt tolerant than

wheat. The yield of wheat was maximal at 1.3 gm. NaCl/1. of soil solution (calculated for the saturation percentage of the soil, 40%) and declined to 50% of maximum at 7.6 gm. NaCl/1. Yankovitch and Boeuf (219) proposed that the toxic level of NaCl for wheat in gm./kg. dry soil could be obtained by multiplying the wilting point, expressed in gm. water/100 gm. dry soil, by 0.04.

Ota, Ogo and their associates have made a series of studies on the effect of salinity on wheat (157, 158, 159). They found that vegetative growth was markedly inhibited in plants which were injured by saline irrigation water during the early growing period, and that very serious injury occurred in plants which were irrigated with saline water at the boot stage (ear-formation period). In all cases, increasing the salt content of the irrigation water inhibited root growth.

Reeve, Allison and Peterson (170), working in the Delta area, Utah, found that yields of wheat varied inversely with the residual salinity of the soil. At salt levels below 15 mmhos. (EC_e), slight reductions in salt content resulted in large increases in yield. Average yields of from 41 to 43 bushels per acre were obtained on plots at three experimental sites leached with four feet of water, compared to yields of one, five and 29 bushels per acre where no leaching water was applied.

Rice can be regarded as only moderately resistant to salt, but its sensitivity may vary with variety and the stage of development at which salinity is applied. Cerighilli and Durand (40) point out that the most sensitive stages are during tillering and flowering. Other facts to consider are the temperature of the medium, although this has not been too critically studied, pH, the composition of the medium, and aeration. Greenhouse studies by Kapp (114) indicated that addition of 5700 p.p.m. NaCl on a dry-soil basis prevented grain formation but resulted in only a slight decrease in the production of straw. The rate of germination was definitely reduced when salt was applied in the field prior to seeding. His studies indicated that the rice plant is most sensitive during the germination stages and is more resistant if salt is not applied until after the plants are six weeks old. Peevy (165) has found that a salt concentration of 2500 p.p.m. in the irrigation water damaged rice severely, regardless of the age of the rice when the salt was added.

All workers on the salt tolerance of rice have observed that there are

great differences in response, depending upon the stage of the life-cycle at which the salinity is introduced. This is well illustrated by the work in Japan by Iwaki, Otoa, Shimose and others (105-109, 161, 162, 181-183). For example, vegetative growth was markedly inhibited for a time in plants which were injured by saline irrigation before the "middle growing stages." Heading and ear-length were markedly reduced in plants irrigated at the earlier and middle stages of ear formation (boot stage), while irrigation in the heading stage resulted in serious reduction in the number of fertilized grains per head. Iwaki and Ota (107, 108) found rice to be sensitive in the germination stage, but germination was almost normal with a sodium chloride concentration up to 0.06 to 0.1 percent.

Iwaki (105) described the effects of brief two-day applications of sodium chloride solutions to rice plants in sand cultures. A 1% salt solution retarded germination, and 2.5 to 3% caused complete inhibition. Susceptibility to salt injury increased as the plants grew older. Elongation, and therefore height, of plants was least affected, the tillering stage was somewhat more sensitive, and the plant was most susceptible during the boot stage. Greater damage occurred from treatment during the day than at night. Osmotic effects apparently predominated, inasmuch as sodium chloride, dilutions of sea water, potassium nitrate and sucrose were equally effective at isosmotic concentrations.

Del Valle and Babe (60), in their studies of the tolerance of rice to sodium chloride, also report that the response of the plant varies with age. They found that a concentration in the soil moisture of 0.15 and 0.25% sodium chloride affected the yield of rice plants 30 and 60 days of age, respectively. A concentration of 1.0% sodium chloride apparently did not affect the production of rice plants which were 90 days old.

Of the fiber crops, cotton is very salt tolerant (99, 203). Wadleigh, Gauch and Strong (210) found that cotton roots can easily penetrate soil containing 0.25% sodium chloride where the critical osmotic pressure ranges from 16 to 17 atmospheres. Recent field-plot studies at the U. S. Salinity Laboratory indicate little or no reduction in yield of seed cotton with salinities up to 6 millimhos (EC_e), although vegetative growth is markedly restricted. A progressive decline in yield occurs at higher salinity levels, reaching 50% of maximum yields at approximately 16 mmhos. (EC_e). The salt tolerance of a number of

upland varieties tested was essentially the same. Asghar and Hamid (5) have studied the use of cotton and sugar cane on saline or partially reclaimed soils and have found that it is possible to decrease the salinity of soil during the cultivation of cotton more quickly than can be done with sugar cane, provided the cotton is grown under more abundant irrigation than is normally used.

Strogonov and Ivanitskaia (193) report that salinization, especially with chloride, increases the stability of the chlorophyll-protein complex in cotton leaves except that in salt-injured leaves a decrease in leaf protein results in a lower stability of the chlorophyll complex. Strogonov et al. (194) claim that salinization with a predominance of sulfate is less deleterious to the growth of cotton than when chloride predominates. Leaf temperatures were differentially affected by these treatments, suggesting varied effects on transpiration. Semergei (180) reported cotton to be moderately tolerant to exchangeable sodium with maximum yields at 4.1 to 6.9% exchangeable sodium. The higher level of exchangeable sodium delayed maturation. At 47% exchangeable sodium the cotton did not mature. Cotton was, therefore, stated to be less tolerant to exchangeable sodium than oats, wheat and other grain crops.

All investigators have regarded sugar beets as having very good tolerance, provided a stand can be obtained (17, 78, 184, 203, 218). Wadleigh, Ayers and Bower (206) found sugar beets to be highly salt resistant and report a 50-percent decrement in yield at EC_e values ranging from 10 to 20 mmhos. Bower and Wadleigh (30) found sugar beets to be very sodium tolerant in addition to being very salt tolerant.

Asghar and Hamid (5) observed that sugar cane was very sensitive to salt during the starting of the crop, and pointed out that it is essential that sugar cane cultivation be carried out on reclaimed areas or those from which the salts are well leached out of the top foot of soil. Narasimhan and Dutt (152) report that sodium chloride concentrations of 0.05 to 0.20% affected the development of sett roots in sugar cane with appreciable varietal differences in sensitivity to salt.

Corn is moderately salt tolerant (203), and the same is true of sorghum. Yankovitch (217) reports a 20% decrease in the yield of corn at 4.1 gm. NaCl/1. in the soil solution compared to equivalent reductions in yield of beans at 2.5 gm. NaCl/1. Corresponding values for millet and sorgho were 4.7 and 7.7 grams, respectively, indicating

appreciably greater tolerance for sorgho than corn. Wadleigh, Gauch and Strong (210) found Mexican June corn to be less tolerant than alfalfa, and more tolerant than beans.

Hayward and Spurr (98) have established that flax is moderately salt tolerant. Using sand-culture techniques under greenhouse conditions, Punjab flax was tested with sodium chloride, calcium chloride and sodium sulfate. At high concentrations, 3.5 and 4.5 atmospheres, relative yields of seed were reduced 26 to 62 percent, and no mature seeds were produced at the highest concentration of sodium sulfate. Simonneau (184) lists flax as being moderately salt tolerant, and Grillot (78) regards it as being in the group of plants that is moderately sensitive to salt. Yankovitch (218) gives the 20% yield decrement value for flax as 8 gm. NaCl/1. of soil solution.

Lehr and Wybenga (131) carried on pot experiments to determine the sensitivity of flax to sodium and reached the conclusion that sodium has a favorable effect on the development and quality of flax-straw. They found that sodium stimulated vegetative development, even in the presence of an ample potassium supply, and that the effect on the fiber was favorable when sodium constituted six percent of the exchangeable cations. Above that point, the quality of the fiber gradually decreased.

Investigations by Retzer and Mogen (173) and Wadleigh, Gauch and Magistad (209) indicate that guayule has medium salt tolerance. The former investigators observed that guayule grew well where the salt concentration did not exceed 0.3 percent in the five-foot profile. Growth was increasingly retarded as salt concentrations in the surface two feet increased to 0.6 percent, and the plants were usually killed or did not grow following transplanting, when salt concentrations were 0.6 percent in the first or second foot. Wadleigh et al. (209) found that the percentage of rubber increased with increasing moisture stress at the lower ranges of salinity but decreased with increasing moisture stress at the higher ranges of salinity.

It is generally agreed that field beans are among the most salt-sensitive crops (17, 78, 184, 203, 218).

Little is known regarding the salt tolerance of tobacco except for the harmful effect of chloride on quality. Lal and Tyagi (126) report tobacco to be sensitive to boron, toxic effects being observed if boron exceeded one p.p.m. in the substrate.

SALT TOLERANCE OF VEGETABLE CROPS

There is a wide variation in the salt tolerance of vegetable crops (99). Among those showing high salt tolerance are many of the chenopods (garden beets, kale, spinach) and asparagus. At the other extreme, vegetables with very low salt tolerance include beans, celery and radish. Peas are also regarded as rather salt sensitive. Between these two limits, the majority of the vegetable crops have moderate to low salt tolerances. In the approximate order of decreasing salt tolerance, some of them are tomato, most of the cruciferous crops (broccoli, cabbage, cauliflower, Brussel sprouts), sweet corn, lettuce, potato and onion. Lists giving the relative salt tolerance of the vegetable crops have been prepared by Dorsman and Wattel (61), van den Berg (16, 17), van Dam (57), Grillot (78) and the U. S. Salinity Laboratory (203). With few exceptions, the lists are in good agreement with respect to the order of salt tolerance, even though the criteria which have been used to determine tolerance have varied.

Most of the classifications, however, are incomplete, and the relative salt tolerance of species is approximate. It has been pointed out by Eaton (65) and others that there is no particular salt level which is critical in retarding plant growth, and growth depression is usually a progressive condition which increases as the salt concentration of the substrate increases. Another point of interest in regard to the salt tolerance of vegetable crops is that most of them exhibit reduction in rate of growth under marginal conditions of salinity without showing other visible symptoms of injury (94). Some vegetables do exhibit specific symptoms, especially in the later stages of the growth cycle. For example, Bernstein and Ayers (19) have noted marked burning of bean leaves. Many leaves become chlorotic and arch upward; and, ultimately, a number of plants die as a result of over-salinization.

Similarly, onions (20) have been observed to develop leaf burn at moderately high levels of salinity. On the other hand, Imazu and Osawa (104), working with Welsh onion, observed no specific symptoms of injury except dwarfing. These authors found that Chinese cabbage on NaCl treatments developed dark-green curled leaves. In some cases a saline substrate may decrease the yield but improve the quality. For example, soil salinity in the range 2.6 – 7.0 mmhos. (EC_e) caused a marked progressive increase in sucrose concentration of carrot roots and pronounced improvement in flavor (21); but this

improvement in quality was achieved at the expense of reductions in yield of 28 to 82%.

Studies on the salt tolerance of different varieties of a given vegetable crop have shown, in many cases, no significant varietal differences. Bernstein and Ayers (21) studied five varieties of carrots and found that, although there was a definite decrease in yield due to salt at an EC_e of 2.6 mmhos., there were no significant differences in the mean total yields between varieties except under high-salt treatments. Similarly, with lettuce, Ayers, Wadleigh and Bernstein (13) could not detect any significant differences between varieties. On the other hand, although beans in general are very salt sensitive, Bernstein and Ayers (19) observed significant differences between varieties at different salt levels. On salinized plots, Giant Stringless Greenpod, Ranger and Tendergreen varieties gave significantly better yields than Logan and Asgrow Black Valentine. However, all varieties were so sensitive that varietal differences were of little or no practical significance.

A considerable amount of work has been done with respect to the salt tolerance of tomatoes, and the concensus is that this vegetable is moderately tolerant. McNaught and Houston (142) stated that the critical conductivity of the saturation extract for glasshouse tomatoes was between six and seven mmhos. Van den Ende (66), also working with tomatoes under glass, found them to be moderately salt tolerant. Hayward and Long (95) stated that the height of stems and fresh- and dry-weight of tomato vines were reduced as the concentration of the culture solution was increased, and Yankovitch (217) determined that the lethal dose of NaCl for tomatoes was 5.60 gm./kg. — about twice that for haricot beans. The data indicated that the osmotic concentration of the substrate was the primary factor in growth inhibition. Kling (115) studied germination of tomato seeds on salinized substrates in Petri dishes. Solutions of 0.1 M hindered germination, a few seeds germinated at 0.2 M, but none at 0.5 M. He also studied growth in salinized soil at concentrations of 0.05, 0.15 and 0.3 M, and noted a progressive stunting of growth, reduction in the aerial mass of the plants, and gradual decrease in yield as salinity increased.

Novikoff (153) studied the salt tolerance of a variety of vegetable crops in soil cultures salinized with NaCl. Expressing salinity in terms of grams NaCl per liter in the soil solution, he found that low levels of salinity (3 to 5 gm./l.) increased the yields of some crops, notably

tomatoes, pimentos, beets and carrots, but that yields progressively declined at higher salinity levels. Beans were most sensitive, dying at concentrations of five to six gm./1. Lettuce, eggplant and pimento yields declined rapidly to approximately 50% of the controls at salinities of four to six gm./1. Carrots tolerated up to eight gm./1. quite well, but yields rapidly declined in the range of eight to ten gm./1. Onions, beets and tomatoes were the most tolerant crops. High salt tolerance of onions is not confirmed by other investigators (20).

According to Yankovitch (217, 218), a 20% decrease in yields of a number of vegetables occurred at the following salinity levels (grams NaCl per liter in the soil solution): peas, 1; beans, 2; haricot bean, 2.5; corn, 4.1; pimento, 4.3; eggplant, 4.5; onions, 5; colza, 11; kohlrabi, 18; beets, 27.

Most crucifers are reported to have medium salt tolerance (56, 203). Species tested include cabbage, broccoli, Brussels sprouts and cauliflower. Van Dam (57) observed that, while autumn cauliflower had relatively good salt tolerance, spring-grown cauliflower did not produce as well and appeared to be more salt sensitive.

Peas are considered to be rather sensitive (78, 203). While potatoes are more tolerant, van den Berg (16) reported that quality was impaired at relatively low salinity values. Early potatoes appear to be quite salt sensitive (57), but the White Rose variety is moderately tolerant (22). In this case, salinity hastened maturation and did not affect the quality of the tuber. There were no visible symptoms of toxicity, and the reduction in yield was apparently associated with a physiological scarcity of water. Blodgett and Snyder (29), however, report that addition of high amounts of soluble salts affected the appearance and shape of the Russet Burbank potato.

SALT TOLERANCE OF FRUIT CROPS

The salt tolerance of fruit crops has received considerable attention in recent years, and lists showing their relative tolerance have been published by the U. S. Salinity Laboratory (203), Beeftink (15), Dorsman and Wattel (61), Grillot (78), Oganesian (155) and others. Oganesian has classified his fruits under five groups, using the classification set up by Kovda (121). He indicates olive, pomegranate and jujube as most salt tolerant, the eastern persimmon as least so. In general, the various investigators have been in rather good agreement with

respect to the relative salt tolerance of fruit crops. However, it should be borne in mind that their classifications are incomplete, and, as Grillot (78) points out, authorities are not always agreed regarding some species. Differences with respect to some of the interacting factors, e.g., climatic variables, which have a significant impact on the responses of plants grown on saline substrates, may account for apparent discrepancies.

The date palm, mulberry, olive, pomegranate and jujube appear to have the highest salt tolerance, while the most sensitive fruits include papaya, avocado, loquat, persimmon, blackberry and gooseberry. Among the fruits having relatively poor salt tolerance are the pear, apple and most of the common stone fruits. Citrus species are fairly sensitive to salt, lemon being more sensitive than orange or grapefruit.

Magistad and Reitemeier (144) have reported fair growth of dates in the Coachella Valley, California, where the osmotic concentration of the soil solution at a moisture content approaching the wilting percentage was about seven atmospheres. Other workers also regard the date palm to be one of the most resistant of all fruit crops (78).

Grillot (78), Beeftink (15) and Dorsman and Wattel (61) agree that grapes grown out-of-doors are quite salt tolerant, while van den Ende (66) reports that the sensitivity of grapes to sodium chloride was fairly high when the crop was grown under glass in The Netherlands. Ravikovitch and Bidner (169), working with grapes in Israel, found that Chasselas and Muscat Hamburg grapes deteriorated and died when the accumulation of chloride in the leaves ranged from 1.5 to 3.8 percent. The symptoms of injury, in this case, were tip-burn of the leaves and the appearance of yellow and brown spots all over the leaf surface. There was an increase in the rate of drying out of leaves; and, on the severely affected vines, leaves turned brown and dried until they crumbled. In very severe cases, the tips of shoots shrank, changed color from green to dark brown, and eventually died. Bagdasarashvili (14) has noted that the salt tolerance of grafted and self-rooted grapevines increased with age. Sodium chloride in the soil at concentrations above 0.1 percent on a dry-weight basis was harmful to yearling grafts, and vines could not grow in saline soil containing 0.8 percent salt on a dry-soil basis.

Woodham (215) reports a detailed study of chloride accumulation in Sultana grapevines in relation to yield. Petiole chloride was the

best index of chloride status of the vine. Chloride accumulation is greatest in basal leaves and decreases toward the apex. Chloride accumulation in leaves increases with progression of the season. Petiole chloride contents of 1.9% in early December (in Australia) or 2.5% in January were associated with severe burn of basal leaves, restricted growth and poor yields. Two percent chloride in the petioles in December was associated with 0.1% chloride in the canes in the winter-dormant condition and approximately a 50% reduction in yield. Sodium in the petioles was closely correlated with chloride levels but sulfur content showed no correlation. Boron-excess symptoms were also noted, but unlike chloride the boron accumulation was primarily in the leaf blade (up to 400 p.p.m.), not in the petiole (44 to 85 p.p.m.).

Hayward, Long and Uhvits (96) found that, at equal osmotic pressures, chlorides induced greater reduction in yield of peaches and were more toxic than sulfates. Regardless of the salt used, increase in concentration resulted in decreased vegetative growth or in death when the osmotic pressure of the substrate solution exceeded three atmospheres.

Brown, Wadleigh and Hayward (35) studied the response to salinity of six varieties of stone fruit trees, using a sand-culture technique. They found that plum and Texas almond were more sensitive to sodium than apricot, peach, prune and Nonpareil almond. There was also a differential response to specific salts which appeared to be more important than the effect of osmotic concentration. For example, calcium chloride was more toxic at isosmotic levels than sodium chloride. The calcium ion apparently facilitated the entry of chloride.

Bernstein, Brown and Hayward (23) compared growth and chloride accumulation in stone-fruit trees on various rootstocks. Yunnan root compared to Lovell greatly increased chloride accumulation and depressed growth of apricot, while Marianna root compared to Lovell effectively restricted chloride accumulation and improved growth in the plum and prune. The Shalil root tended to increase chloride accumulation in peach and almonds. Under the conditions of the experiment, half of the overall growth reduction in the trees on the Lovell roots in the saline soil was attributed to toxic effects of chloride accumulation and the remainder of the growth depression to osmotic effects of the saline soil.

Hansen (89, 90) determined the effect of rootstock on boron accu-

mulation and toxicity in plum, prune, almond and peach trees. In the experiment with French prune, the ranking of rootstocks in order of decreasing boron accumulation and injury in the scion was: Shalil peach root, Marianna, apricot, myrobalan and almond. Similar results with selected rootstocks from this list were obtained for the other scions tested. As with citrus, the predominant influence of rootstock on boron accumulation and toxicity was apparent. Woodbridge (214) describes boron toxicity symptoms in stone-fruit trees.

Lilleland, Brown and Swanson (133) showed that sodium may cause leaf-tip burn in almonds. The symptoms of injury begin most commonly at the tip of the leaf, and later the entire leaf may scorch severely and fall prematurely. They found that there was a slight leaf-tip burn when the sodium percentage in the almond leaf on a dry-weight basis ranged from 0.3 to 0.6 percent. Moderate injury occurred from 0.6 to 1.3 percent, and severe injury above that level of sodium accumulation.

Chapman (42) stated that sodium or chloride accumulation in orange leaves, three to seven months old, was excessive if it was greater than 0.25% on a dry-weight basis. Pearson and Goss (163), working with young grapefruit trees, found that high salt concentrations in the irrigation water were associated with bronzing and burning of the leaves. With soil salinities of six to eight mmhos. (EC_e), there was defoliation of the young trees. Further work on grapefruit indicated that there was an inverse relationship between the fresh weight of tops or the increase in bole diameter and the conductivity of the saturation extract. Water-table conditions affected growth primarily by increasing the EC_e of the soil associated with given irrigation-water qualities (164).

Cooper and his associates in Texas (45, 46, 48, 49, 55) have worked intensively on the salt tolerance of citrus varieties, especially with reference to the effect of rootstock in conditioning the accumulation of chloride. In a study of 20 rootstocks, they found that the Cleopatra mandarin, *Severinia buxifolia* and Rangpur lime rootstocks appeared to be the most resistant to chloride accumulation and salt injury (49). Sodium accumulation was lowest for the sweet oranges, next for trifoliate orange hybrids, and highest for mandarins.

Rootstock affects other aspects of mineral nutrition in addition to chloride accumulation. Cooper, Gorton and Olson (50) studied the influence of salinity and excess boron on mineral composition of

Valencia orange and Shary Red grapefruit on Cleopatra mandarin and sour orange rootstocks. The dominant influence of rootstock on chloride accumulation was evident, both scions accumulating comparable quantities of chloride, the concentration being, however, about twice as great for each scion on the sour orange rootstock as on the Cleopatra mandarin. The grapefruit scion, however, accumulated more sodium than the orange. Accumulation of calcium and boron was greater on the Cleopatra mandarin than on the sour orange root, while potassium showed the reverse relationship. The pronounced effect of rootstock on calcium and potassium nutrition was believed to be without appreciable effect on salt tolerance (77) because grapefruit on Cleopatra mandarin rootstock was more tolerant than on sour orange, despite the greater Ca and lower K uptake.

Rootstock exerts a predominant influence on boron accumulation as well as on chloride (80). In a study of 45 rootstocks on soils irrigated with high-boron water, Cooper, Peynado and Shull (54) reported that sweet lime and sweet lemon roots caused high boron accumulation, while Cleopatra mandarin, sour orange, sweet orange and rough lemon roots caused lower boron accumulations. *Severinia buxifolia* roots gave the lowest boron accumulations. Orange and grapefruit scions on the same rootstocks had very similar boron accumulations.

Cooper, Olson, Maxwell and Otey (52) concluded that no single known rootstock combined maximum tolerance to the three soil factors, salinity, excess boron and lime. Rangpur lime came closest to the ideal, with good salt and lime tolerance, and moderate boron tolerance. *Severinia buxifolia* has good salt and boron tolerance but poor lime tolerance, while sour orange and rough lemon have good lime tolerance but only moderate salt and boron tolerance. Cleopatra mandarin has good salt tolerance, moderate lime tolerance, and almost moderate boron tolerance.

In a study of sodium accumulation in Lisbon lemon trees on sour orange roots, Haas (85) found that sodium in the bark and roots of the trees and in the peel and pulp of the fruit was more closely correlated with sodium concentration in the applied solution than was the sodium content of the leaves. Jones, Martin and Bitters (110) studied the influence of exchangeable sodium and potassium on the growth and composition of Allen Eureka lemon on seven different rootstocks. The reduction in growth at comparable levels of exchangeable sodium was

greater on Yolo loam with a cation-exchange-capacity of 19 milliequivalents per 100 grams than on Yolo sandy loam (cation-exchange-capacity equal to 10 milliequivalents per 100 grams). Martin and Bingham (146) have reported a similar effect of cation-exchange-capacity on the tolerance of avocado to exchangeable sodium. While rootstock exerted an effect on sodium uptake and cationic composition of the lemon scion, Jones, Martin and Bitters (110) concluded that these compositional effects were not related to differential growth of the trees.

Jones et al. (111) report that continued use of sodium nitrate without lime in a long-range fertility trial with Washington Navel orange trees resulted in a depression of yields. Increased percentages of sodium in the irrigation water in another experiment resulted in more marked increases of sodium in the root xylem than in root bark or leaves.

Haas and Brusca (87) report that reciprocal variations of sodium and calcium in nutrient solutions caused decreases in growth of rooted sweet orange seedlings with increasing proportions of sodium. Potassium tended to increase and calcium to decrease in the plants as the sodium in the solution increased. Calcium content was not appreciably affected in soil cultures with increasing levels of added sodium nitrate.

Haas and Brusca (86) determined the influence of increasing levels of sodium applied to soil cultures on the growth and composition of citrus seedlings. There appeared to be significant differences in sodium accumulation, ranging from less than 0.1% for most seedlings to 0.3% for tangelo. Sodium in the roots was lowest in Troyer citrange (0.14%) and highest in sweet orange (0.5%). Haas (81) described leaf injury patterns for chloride and boron accumulation in citrus leaves and compared these with phosphorus- and potassium-deficiency patterns. Chloride burn progresses more rapidly along the leaf margin than the midrib, while the reverse is true of potassium and phosphorus deficiencies. Boron toxicity results in a burned tip with a straight margin perpendicular to the midrib.

Zusman (221) studied the effect of increasing sulfate concentrations, instituted for pH control, on the growth and composition of sweet lime and sour orange seedlings. A sandy citrus soil adjusted to 22% exchangeable sodium was used. Sulfate toxicity symptoms, characterized by a yellowing of leaf margins spreading interveinally toward the midrib and followed by necrosis, developed in the sweet lime seedlings

within eight days after planting. Similar symptoms appeared in the sour orange seedlings ten days later. The SO_3 content of leaves increased to about 1%. While calcium tended to decrease with increasing levels of sulfate, no deficiency levels for calcium were reached. Sodium toxicity symptoms developed about 50 days after planting in all sweet lime seedlings. The leaves contained 0.20 to 0.28% sodium. The leaves of sour orange seedlings contained only 0.07 to 0.13% sodium.

Sodium in citrus was also studied by Rodney and co-workers (174, 175) as a possible factor in lemon tree collapse. They concluded that increased sodium was the result, not the cause, of sieve-tube necrosis which was the immediate cause of lemon tree collapse.

Ayers (7) found that the avocado is very sensitive to accumulation of chloride and sodium ions in the leaf. Even with relatively low concentrations of salt in the soil solution, injurious amounts of chloride or sodium may be accumulated. Ayers, Aldrich and Coony (10) state that avocado leaves which are free from chloride burn seldom contain as much as 0.5% chloride on a dry-weight basis. One-tenth of the leaf-blade may be affected when the chloride content ranges from 0.5% to 0.7%, and there is greater involvement of the leaf-blade as the amount of chloride increases. Haas (82) also observed that excessive chloride accumulation in avocado leaves results in tip burn and that severe leaf burn occurred with 12 meq./1. of chloride in the culture solution. These studies were made with calcium chloride as the added salt. In a series of soil-sand cultures with added sodium chloride, he noted that chloride accumulation was much less in West Indian seedlings (var. Waldin) than in Guatemalan (var. Hass) or Mexican (var. Topa Topa) seedlings. Haas and Brusca (88) compared chloride absorption in five varieties of Mexican and six varieties of Guatemalan seedlings. Chloride accumulation in the leaves of Mexican seedlings was 50 to 75% greater than in Guatemalan varieties, and the Mexican seedlings also accumulated more chloride in the roots.

Cooper and Gorton (47) confirmed the effect of rootstock on chloride accumulation, finding less chloride accumulated in avocado on West Indian roots than on Mexican. On a Mexican root, Mexican scions accumulated more chloride than Guatemalan or hybrid scions. On West Indian root, however, Cooper (43) found greater chloride accumulation in Lula, a hybrid variety, than in Y9 (Mexican race). Comparing chloride accumulation in avocado to that in grapefruit in

a parallel experiment, Cooper and Gorton (47) report more rapid chloride accumulation and evidence of damage at lower chloride contents in avocado leaves than in grapefruit. The avocados tended to accumulate more chloride but less sodium than did grapefruit.

Haas (83) studied sodium injury in avocado seedlings as a function of reciprocally varied calcium and sodium concentrations in sand cultures. Sodium burn of leaves occurred with 27 meq./1. of sodium, the older burned leaves containing more than 1% sodium, the younger leaves ranging from 0.14 to 0.56%. Relatively little sodium was found in roots, injured plants containing 0.25 to 0.32% sodium on a dry-weight basis. Haas (84) also studied varietal differences in sodium accumulation and injury. Seedlings of the Mexican varieties, Mexicola and Harmon, contained up to 0.9% sodium in the leaves and burned severely with 200 p.p.m. sodium in the culture solution. Two other Mexican varieties, Topa Topa and Caliente, however, showed little or no injury, with only about 0.1% sodium in the leaves. Anaheim seedlings (Guatemalan race) showed no leaf-injury symptoms and contained only 0.04 to 0.05% sodium in the leaves. Sodium accumulation in roots was not closely correlated with accumulation in the leaves, ranging from 0.3% in Mexicola roots to about 1.0% in Topa Topa. Anaheim, in which restricted translocation of sodium to the leaves was observed, contained 0.7 to 0.8% sodium in the roots. Cooper (43) recorded greater sodium accumulation in Y9 scion (Mexican race) on Mexican root than on West Indian root.

Wallace et al. (211) compared sodium and chloride accumulations in Fuerte avocado scions on *Persea floccosa* rootstock with three *Persea americana* rootstocks representing the West Indian, Guatemalan and Mexican avocado races. *Persea floccosa* rootstock produced higher sodium and chloride contents in the scion 180 days after NaCl application to the soil than any of the three *P. americana* rootstocks.

Several investigators have reported that the strawberry is very sensitive to salt. Brown and Voth (32) indicated that, when water containing more than 100 p.p.m. sodium chloride was used to irrigate strawberries by the furrow-irrigation system, there was sufficient salt accumulation to produce a measureable decrease in the yield of berries. This may occur although the plants themselves do not show visible signs of injury. They found that a change from furrow irrigation to a system of sprinkler irrigation may effectively reduce salt accumulation, with a

resultant higher yield of fruit, especially during the second year of growth. Van Dam (57) places strawberries as next to the lowest in salt tolerance of the economic plants which he studied, and Grillot (78) classifies strawberries in the "salt-sensitive" category. Imazu and Osawa (104) list strawberries as very salt-sensitive and report that the plant showed severe leaf-scorch, a blackish-brown root, and poor growth in a solution containing 3000 p.p.m. of NaCl.

Papaya has very low salt tolerance. Data obtained by Cooper (44) indicate that papaya roots accumulate large amounts of chloride when grown in saline soils. Some of the chloride is transported to the foliage, and there is resultant leaf injury, but a large amount of the chloride is retained in the roots. He found that mature necrotic leaves showed above 1.0% chloride on a dry-weight basis, and there was an even greater concentration of chloride in the roots. Cooper and Peynado (53) studied sodium and chloride accumulation in papaya plants grown in the field with soil salinities in the saturation extract of two mmhos. or less. Despite the low level of soil salinity, large accumulations (up to 5%) of both sodium and chloride were found in roots, trunks and petioles of the trees, but relatively restricted accumulation was noted in the leaf-blades. Because of the almost equivalent accumulation of sodium and chloride, the injury could not be specifically attributed to either ion.

In a report of salt tolerance of subtropical fruit plants, Cooper and Gorton (47) note that white sapote, mango and rose apple evidence leaf-burn symptoms when chloride accumulations in the leaves are in the order of 0.4 to 0.9%. Cooper, Cowley and Shull (45) indicate the maximum salinity ranges at which no leaf-burn develops as follows (EC_e in mmhos.): citrus, 7.5 to 1.5, depending mostly on rootstock; natal plum, 4 to 2; white sapote, papaya, mango and cherimoya, 2 to 1; avocado, 1.5 to less than 1.

The specific sensitivity of many fruit trees to sodium and chloride is reflected in their low tolerance to these ions when the foliage is wetted by a sprinkler system. Harding, Miller and Fireman (91) have reported extensive leaf-burn symptoms in sprinkler-irrigated citrus groves, including orange, grapefruit and tangerine. They found up to 0.6 % sodium or chloride in the sprinkler-wetted leaves of citrus compared to only 0.04 to 0.17% of these elements in unwetted leaves of the same trees. The irrigation waters used in the warm interior

valleys of California where this damage occurred contained 75 to 131 p.p.m. chloride and 69 to 190 p.p.m. sodium. Older sprinkler-irrigated citrus areas in which this difficulty has not developed apparently benefit from use of less saline waters and lower evaporative conditions. Personal communications and unpublished observations indicate that stone fruit trees are even more susceptible to this type of injury than citrus, while avocado is relatively insensitive. The various degrees of sensitivity appear related to the rate of foliar absorption of chloride and sodium.

FORAGE PLANTS — GRASSES AND LEGUMES

Forage plants—grasses and legumes—include some very salt-tolerant species and also some very sensitive ones. Hayward and Wadleigh (99) and Hayward (93) have reviewed the early work done on the salt tolerance of this group in the United States. The U. S. Salinity Laboratory (203) has reviewed previous work and summarized the experimental work at the Laboratory with respect to the relative salt tolerance of the forage crops, expressing the degree of salt tolerance in relation to the electrical conductivity of the saturation extract of soil in millimhos per centimeter (EC_e).

Among grasses which have shown high salt resistance in the United States are alkali sacaton *(Sporobolus airoides)*, saltgrass *(Distichlis stricta)*, Nuttall alkali grass *(Puccinellia nuttalliana)*, Bermuda grass *(Cynodon dactylon)* and Rhodes grass *(Chloris gayana)*. Western wheatgrass *(Agropyron smithii)* is also salt-tolerant. These grasses are able to tolerate an EC_e between 8 and 12 mmhos., and some of them may survive when the EC_e goes as high as 16 or 18. Less tolerant forage grasses are orchard grass *(Dactylis glomerata)*, blue grama *(Bouteloua gracilis)*, meadow fescue *(Festuca elatior)*, reed canary *(Phalaris arundinacea)* and smooth brome *(Bromus inermis)*. These grasses produce fair yields in the range of four to eight mmhos. One of the least tolerant grasses is meadow foxtail *(Alopecurus pratensis)*.

It should be noted that the relative salt tolerance of grasses may depend, to a high degree, on other factors of the environment. For example, studies in Riverside, California, indicated that the response of some grasses to salinity may be greatly modified by high soil temperatures. This is the case for reed canary grass, perennial rye grass *(Lolium perenne)*, meadow fescue and orchard grass. Gauch and Wad-

leigh (73) investigated the salt tolerance of Rhodes grass and Dallis grass *(Paspalum dilatatum)*, and found that, on a relative basis, the Dallis grass was much less tolerant than Rhodes grass, and also that the latter produced more forage on an absolute basis. They interpreted the salinity responses on the basis of the physiological availability of water, but noted that the sodium bicarbonate treatment was exceedingly toxic to Dallis grass. Working with the same grasses, Bower and Wadleigh (30) observed that Rhodes grass was very sodium-tolerant. Dallis grass was not too seriously affected by exchangeable-sodium-percentages of 30 or less, but no appreciable growth occurred at higher sodium levels.

Gausman, Cowley and Barton (74) studied the relative salt tolerance of five grasses grown in Texas, by adding 1500 to 4500 p.p.m. $CaCl_2$ and NaCl to the irrigation water. The five grasses exhibited salt tolerance in the following decreasing order: Rhodes grass; coastal Bermuda; blue panic *(Panicum antidotale)*; buffel *(Pennisetum ciliare)*; Angleton *(Andropogon nodosus)*.

Forsberg (69) conducted field tests on six different alkali areas in Saskatchewan, Canada, and included in the tests common species of grasses, legumes and cereal crops. He also conducted greenhouse tests, using natural saline soils taken from the field-test areas. His results indicate that slender wheatgrass *(Agropyron trachycaulum)* and tall wheatgrass *(A. elongatum)* were the most resistant of all crops tested. The former was somewhat better under dry conditions and the latter under moist. Other findings were that Macoun's wild rye was about equal to slender wheatgrass and wild barley in its tolerance. Moderately resistant grasses included Achenbach and Commercial brome grass, Ree intermediate wheatgrass, Fairway crested wheat, Canadian wild rye and Russian wild rye. The most salt-sensitive crops were the Western couch grass, Standard crested wheat, and some of the annual crops tested. McKenzie and Bolton (141), also working in Saskatchewan, tested a wide range of crops and found the best adapted ones among the grasses to be slender wheatgrass, brome grass, crested wheatgrass, meadow fescue and reed canary.

In Australia, Millington, Burvill and Marsh (151) carried on exploratory salt-tolerance tests with several grasses, using various dilutions of sea water to salinize soils to a range of 0.02 to 0.3% NaCl. They also used soil columns with salt concentrations increasing from 0.05 – 0.1% in the surface to 0.4 – 0.6% in the deeper layers of the profile.

They found that cereal rye showed the greatest germinative capacity, and barley the best growth under saline conditions. *Paspalum vaginatum* exhibited good salt tolerance at salt levels up to one-half to two-thirds sea water, curly rye grass *(Pholiurus incurvis)* and sea barley grass *(Hordeum marinum)* showed good salt tolerance, greater, in fact, than Wimmera rye grass *(Lolium rigidum)*. However, there were difficulties in establishing these species.

In French North Africa, Simonneau (184) and Grillot (78) have noted some grasses which have good salt tolerance. Simonneau indicates that Bermuda grass, *Polypogon monspeliensis*, maritime barley *(Hordeum maritimum)* and fescue *(Festuca arundinacea)* are salt-tolerant forage grasses. Grillot has observed the salt resistance of Rhodes grass and Dallis grass. Among the moderately sensitive grasses, he lists meadow grass and tall fescue.

Relatively little work on the salt tolerance of forage crops has been reported from The Netherlands, but van den Berg and Westerhof (18) have indicated that grass for seed and grass for grazing are relatively salt tolerant, as is winter rye. They found red clover *(Trifolium pratense)* to be very sensitive, especially to inundation by saline waters.

Oganesian (154) carried out rather extensive tests on the salt tolerance of perennial grasses, using laboratory and field techniques, and found considerable variation in relative salt tolerance. Among the most tolerant were *Agropyron repens* and *Beckmannia eruciformis*. Somewhat less tolerant were rye grass *(Lolium multiflorum)* and meadow fescue *(Festuca elatior)*. In the "medium" group he placed orchard grass *(Dactylis glomerata)* and crested wheatgrass *(Agropyron cristatum)*. Grasses which he found to be relatively sensitive included perennial rye *(Lolium perenne)*, wheatgrass *(Agropyron tenerum)*, timothy *(Phleum pratense)* and smooth brome *(Bromus inermis)*.

Repp (172) investigated the tolerance of grasses and legumes and a few cultivated species on saline-sodic soils (szik) of Hungary, and states that, except for some native salt grasses, growth of most species begins to decline at salt concentrations of 0.25% and pH's above 8.0. She lists the species studied in order of decreasing salt tolerance as follows: *Atropis limosa*, *Beckmannia eruciformis*, rice, *Alopecurus pratensis*, *Lolium italicum*, sunflower, corn, *Ricinus*, rape, *Trifolium repens*, white lupine, pea, soybean.

Some of the leguminous forage plants are moderately salt tolerant

(203), including alfalfa *(Medicago)*, white and yellow sweet clovers *(Melilotus alba* and *M. officinalis)*, birdsfoot trefoil *(Lotus corniculatus)*, strawberry clover *(Trifolium fragiferum)* and hubam clover *(Melilotus alba* var. *annua)*. Legumes showing poor salt tolerance include white Dutch clover *(Trifolium repens)*, red clover *(Trifolium pratense)* and Ladino clover *(Trifolium repens* var. *latum)*. Ayers (6), reporting on the salt tolerance of birdsfoot trefoil, found that it was highly salt tolerant, could withstand high summer temperatures, and compared favorably with alfalfa. Birdsfoot trefoil appeared to be better adapted to salinity and high summer temperatures than red, alsike, Ladino and strawberry clovers, and was also superior to big trefoil *(Lotus uliginosus)*. Strawberry clover *(Trifolium fragiferum)* exhibited relatively poor growth at the highest salinity levels, but it is reputed to have the ability to grow in the presence of high water tables.

Gauch and Magistad (71) compared the growth of strawberry clover varieties, alfalfa and Ladino clover and found no evidence that a given concentration of solution may be regarded as critical. Instead, there tended to be a linear relationship between growth reduction and increase in salt concentration of the solutions expressed in atmospheres. On an actual yield basis, alfalfa and Ladino clover produced 1.3 and 1.9 times as much as strawberry clover. On a relative basis, the Nebraska strain of strawberry clover was more salt tolerant than alfalfa or Ladino clover. Five strains of strawberry clover were investigated, and the salt tolerance was in the following descending order: Nebraska, Idaho, Colorado, Washington, Oregon.

Brown and Hayward (33) studied the salt tolerance of several varieties of alfalfa at Riverside, California. California common and India were the best adapted to the climate and produced the best yields at the control and low-salt (EC_e 4.2 mmhos.) levels. Turkestan and Atlantic produced more forage than other varieties at the intermediate (6.6 mmhos.) level, and at the highest salt level (9.0 mmhos.) there was no significant difference between varieties. Average yields were reduced to 79 percent of the control at 4.2 mmhos., 60% of the control at 6.6 mmhos., and 42% of the control at 9.0 mmhos. The osmotic pressure of the soil solution appeared to be the significant factor in the reduction of yields on saline substrates.

Kling (115) tested alfalfa in soil cultures salinized with balanced salt solutions. Growth decreased with increasing salinity, and the leaves

became more succulent and darker green. Nodules failed to develop when 0.3 M salt solutions were used. Photosynthesis was higher in the plants on the saline treatment than in control plants. A similar effect was observed with tomato plants.

Uhvits (201) investigated the effect of osmotic pressure on water absorption and the germination of alfalfa seeds. In all of her studies, the higher the concentrations of sodium chloride or mannitol, the lower were the rate and the percent germination of alfalfa seeds. She found that germination of alfalfa was practically inhibited on sodium chloride solutions of 12 to 15 atmospheres. Differences observed in the response to substrates with isosmotic concentrations of sodium chloride and mannitol suggested that there might be a toxic effect of the sodium chloride which increased with increasing concentration of the salt.

Dunnewald (64), studying the removal of alkali by drainage and leaching, found that alfalfa was more sensitive to salt than sweet clover. He also observed that leaching improved germination of the seed, and his tests indicated that, as the salt content decreased, the germination improved. Ayers and Hayward (12), working with alfalfa and other crops, developed a method for obtaining information on the relative salt tolerance of germinating seeds. They found that there was no general relationship between the relative salt tolerance of germinating seeds and the tolerance during later phases of growth. For example, alfalfa and sugar beets are regarded as salt-tolerant crops but they were found to be relatively sensitive to salinity during germination.

Rogers (176), studying boron fertilization of legumes, noted toxic effects of excess boron with several species. He notes that Austrian winter peas appear to be most susceptible to excess boron, and he lists other species in order of decreasing susceptibility as follows: crimson clover, vetch, bur clover. Injury was most severe on coarse-textured soils at low moisture contents. As little as 15 pounds per acre of borax caused severe injury to Austrian winter peas, crimson clover, red clover and white clover on sandy soils when boron was applied at the time of seeding.

ORNAMENTALS AND FLOWERS

Grillot (78) notes that almost all flowers and ornamental plants are sensitive to salt, and refers to the violet as being completely intolerant of salt. Butterfield (39) has listed the ornamental plants observed in

California which, over a long period of years, have indicated some tolerance to both saline and alkali soils. He states that some of the plants listed have more tolerance than others but does not give any quantitative data in this regard. He classifies plants into several groups, including trees, palms and tree-like plants, shrubs and vines, bulbs and roots, and annuals and perennials. He points out that it is important to consider other factors such as soil texture, fertility, soil management, water supply, drainage and especially climate.

Cooper and Link (51) have prepared a list of subtropical ornamental plants in order of their tolerance under three groups: moderately salt tolerant, poorly salt tolerant, and very poorly salt tolerant. In the first group are, among others, *Sansevieria*, *Bougainvillea*, *Hibiscus* and *Oleander*; in the second group are the orchid tree *(Bauhinia)*, the red-leafed banana *(Musa)*, the Japanese yew *(Podocarpus)* and poinsettia *(Euphorbia)*, while some of the genera in the third, "very poorly salt tolerant," group are *Gardenia*, *Camellia*, *Azalea* and *Magnolia*.

Ferguson (68) has a classification of the plants of southern Florida based upon the ability of the plant to withstand salt spray. She defines tolerant plants as those which can withstand salt spray and an occasional flooding by salt water, and points out that the salt spray on the leaves causes exosmosis and results in burning and defoliation of non-salt-tolerant plants. About 275 plants are listed under headings, such as ground cover, shrubs and herbaceous perennials, and trees. Lothian (135) has prepared a similar salt-spray tolerance list for southern Australia. The work was inspired by Miss Ferguson's paper and supplements or subtracts from her list on the basis of Australian conditions. Westgate (212) states that salt-spray injury to native vegetation on the Florida keys is similar to foliar injury of azaleas and camellias sprinkled with artesian water containing 583 and 918 p.p.m. chloride. When the water was applied to the soil only, instead of the foliage, injury was less severe. This suggests that foliar absorption of chloride or possibly other ions may have aggravated the injury. Tip burn was also stated to occur in mangos and avocados sprinkled with salty well water in south Florida.

Specific investigations on floral plants are relatively limited, many of the papers coming from Lunt, Kofranek and their associates at the University of California at Los Angeles (116-120; 136-139). Among the flowers studied, carnations appear to have won a reputation of being

a crop which is very tolerant to saline conditions. However, recent studies (137) indicate that both the production and the quality of blooms are significantly reduced at moderate concentrations of salts. For example, calcium chloride and sodium chloride at 4200 p.p.m. substantially reduced the production of Sims carnations, while quality decreased gradually, although still of commercial grade, at higher concentrations of soluble salts. They recommend that carnations be grown at a value for the electrical conductivity of the saturation paste of less than 2.5. These investigators also found that carnations were very tolerant of boron and that waters containing 2 p.p.m., or possibly somewhat higher concentrations, would not be objectionable for carnation growing. Van den Ende (66), studying the effect of saline water on the response of crops grown under glass, found that carnations were only slightly sensitive to sodium chloride. The general upper limit of safety for all crops studied was 0.025 of sodium chloride on a dry-soil basis.

Groote (79) gives a maximum permissible level of 500 p.p.m. chloride in irrigation water for sprinkling floricultural crops. He states that *Primula* and *Hydrangea hortensis* are very sensitive to the chloride ion. Roses are also stated to be exceptionally sensitive to salinity, while carnations are more tolerant.

Krupenikov (125) reports that *Clematis orientalis* is highly salt resistant. He found that it would tolerate over 2.0% of total salts, determined on the basis of the solid residue, without showing any signs of inhibited growth. Among the relatively tolerant flowers is *Chrysanthemum.* Kofranek, Lunt and Hart (116) found that chrysanthemums of the Kramer variety exhibited satisfactory growth at an EC_e of six mmhos. Stem length was reduced about 1.7 inches per unit increase in EC_e, but the dry weight was not affected up to an EC_e of six unless specific ion effects were present. When ammonium or magnesium salts were used, stem length was greatly reduced, and high levels of ammonia also markedly reduced the production of dry matter.

Stock *(Mathiola incana)* is moderately salt tolerant. Lunt, Kofranek and Hart (136) studied six varieties under saline conditions and found that the basal leaves became chlorotic when they were grown in a soil with an EC_e of about seven mmhos. At this salt level, the plants were 25 to 30% shorter than those grown at an EC_e of about two. The salt treatments had little effect on the dry weight or flowering date

of the plants. They concluded that the tolerance of stocks to calcium and sodium chlorides appeared to be related to the ability of the plant to accumulate appreciable quantities of sodium and chloride ions without showing deleterious effects.

Asters (120) are moderately sensitive to salinity caused by sodium and calcium chlorides, and exhibit an average sensitivity to excess boron. Plants were grown at 11, 71, 131 and 191 meq./1. Height and flower size were reduced as the concentration of the salt was increased. At the highest salt level, the plants died before the end of the experiment.

Poinsettias as potted plants are relatively sensitive to moderately high salinity or boron levels (117). When the plants were grown at 135 meq./1. of salt ($NaCl + Cal_2$), they abscissed the majority of their leaves. Plant height and the diameter of the bracts were seriously reduced. At boron levels above 4.8 p.p.m., an interveinal leaf chlorosis developed, followed by a marginal scorch and leaf abscission. However, high boron levels had less effect on plant size than did high salinity levels.

Gladioli are also relatively sensitive to moderate levels of salts and boron (118). The plants were tested with treatments of 0, 60, 120 and 180 meq./1. of calcium chloride plus sodium chloride, and also with treatments of boron ranging from two to ten p.p.m. The weight and length of spikes and the number of florets decreased markedly with an increased concentration of salts. Increasing boron up to ten p.p.m. did not have this effect but did cause considerable leaf scorch at the higher concentration. The weight and size of corms and the weight of cormels were reduced as the salt level was increased.

Azaleas have been relatively sensitive to nutritional imbalance and to solutions containing about 50 meq./1. of salt (138). High-bicarbonate waters markedly increased the absorption of sodium when calcium supplies were low, and decreased the absorption of calcium and iron. It appears that, under conditions of low calcium supply, bicarbonate accelerates calcium deficiency.

These same authors (139) found the gardenia, variety Belmont, to be very sensitive to salinity treatments, growth being reduced by 50% or more by a total concentration of about 50 meq./1. in the applied solution. Wilhelm and Pyfrom (213) found that conductivity varied considerably in greenhouse soils where gardenias failed, but

that it was in the range from 2.5 to 5.0 mmhos. (EC of 1:2 soil extract) immediately adjacent to the injured roots. No root damage was observed where conductivities were below 1.5, and the authors indicate that 1.5 to 2.0 mmhos. should be regarded as the upper limit of safety for these flowers when grown on light porous soils.

One of the most sensitive flowers is the African violet *(Saintpaulia)* which showed a decrease in fresh weight and dry weight when a complete nutrient solution containing more than 13 meq./1. total salts was applied to them (119). The plants exhibited a high ability to accumulate sodium, especially in the older leaves. In moderate excess, sodium, chloride, sulfate and calcium ions did not appear to be particularly harmful to the African violet, but the ammonium ion in moderate excess was very much so.

SHADE AND ROADSIDE TREES

Limited work has been done on the salt tolerance of shade and roadside trees. Some studies have been carried out in The Netherlands, Russian investigators have published a few papers, and a few lists have been prepared based on work done in North Africa and the United States.

Butijn (38) in The Netherlands studied trees from the standpoint of their use as wind-screens on saline spots in the Zeeland. He indicates that the osier willow, white poplar, hawthorne and common elm have relatively good salt tolerance when grown on saline spots, while the Italian poplar and black alder are relatively salt sensitive. Another Dutch study, based on the influence of the 1953 flood on tree species, was made by van der Linde and van der Meiden (134). They rated about 30 deciduous trees, showing their relative salt injury on the basis of "C" figures. The symbol "C" was used to indicate the grams of sodium chloride per liter of soil solution, determined at soil moisture contents between 20 and 35%, in the soil layer four to eight inches in depth. Among the most resistant trees were *Lycium halimifolium, Populus alba, P. canescens, Robinia pseudoacacia, Ulmus campestris* and *Quercus rober.* Trees which appeared to be very salt sensitive included *Carpinus Betulus, Fagus sylvatica, Acer Pseudoplatanus, Alnus incana, Populus nigra* var. *Italica, Buxus sempervirens* and *Corylus avellana.* The authors explain that the list is based on observations made during

only one year, 1953, and that it might be subject to alteration as a result of further researches in following years.

In Russia, Zhemchuzhnikov (220) investigated the salt resistance of trees and shrubs near Lake Balkhash. The trees were tested in non-saline soil, soil from a salty spot, and sand from the shore of the lake. Among trees and shrubs which he found to be highly salt tolerant were *Tamarix hispida* and *T. Pallasii, Elaeagnus angustifolia, Robinia, Prunus armeniaca* and *Morus*. Intermediate salt tolerance was exhibited by *Acer Negundo, Pyrus baccata* and *Ribes nigrum*. The least tolerant species included *Betula dahurica, Larix* and *Populus laurifolia*. Other work in Russia includes studies by Krupenikov (123, 124) who has reported on some of the poplars and the aspen growing under natural conditions. He finds that the aspen is especially resistant to salt, as are the white and Canadian poplars. He indicates that *Populus tremuloides* has not been studied as extensively as some of the other species. *Betula kirghisorium* has attained a high degree of salt tolerance through ecological and physiological adaptation.

Gadzhiev (70) reports on the salt tolerance of 55 varieties of shrubs and trees based on observations of growth on saline soil in Baku. Matukhin (149) reported oak seedlings to be more sensitive to chloride than to sulfate in soil cultures.

Grillot (78) lists a number of trees on the basis of their relative resistance to salt and reports that the most resistant species is *Sapindus utilis*, while certain species of poplar, *Eucalyptus (globulus, robusta, occidentalis)* and *Casuarina equisetifolia* are moderately resistant. Walnut was sensitive.

Repp (172) recommends tamarix for strongly saline areas and *Ulmus glabra* and *Elaeagnus angustifolia* for planting on less saline clay soils. For sandier saline-alkali soils, she recommends *Robinia pseudoacacia* and *Populus nigra*.

Little work has been done on the salt tolerance of trees in Japan, but Sakazaki et al. (178) did initiate studies on *Metasequoia glyptostroboides* and compared it, for relative salt tolerance, with corn, tomato, red beet and *Atriplex*. They found that *Metasequoia*, based upon response to an increasing sodium-potassium ratio, was less salt tolerant than tomato and more so than corn.

In the United States, Snyder et al. (187) investigated the salt tolerance of a number of coniferous and deciduous trees in connection with

some alkali reclamation investigations. In general, they noted that the conifers were not successful on alkali soils when the permeability was poor. Among deciduous trees, they found that some of the more salt tolerant included silver poplar, weeping willow, Russian olive and Siberian elm. In all cases they observed that, regardless of the degree of salinization or alkalization, the critical factor was the permeability of the soil. Harper (92) in the United States studied the effects of chloride on pecans and other native trees of Oklahoma and observed that the bur oak, cottonwood and green ash were much less susceptible to chloride injury than pecan, elm and hickory. The pecan and some of the other native trees were injured by chloride concentrations in the soil which would not be harmful to cultivated crops. Pecan leaves developed a brown margin, and the affected trees usually died in one or two seasons. Most of the scorched leaves analyzed in the study contained 1.0% chloride or more on a dry-weight basis. Apparently, depth of rooting was a significant factor because large pecan trees were more susceptible to chloride injury than small trees.

The salt tolerance of roadside trees was investigated by Strong (195) and Traaen (200) to determine the amount of injury to trees caused by calcium chloride when it was used to reduce dust on roads. Strong found that calcium chloride injury was frequent along roadsides where this salt was used, and observed that, while some trees were killed, leaf-scorch was the most common result. The response of different trees to salt injury varied, and he noted that the greatest tolerance to calcium chloride was evidenced by the red and white oaks and the American elm. Those which were least salt tolerant included balsam fir and white spruce among the conifers, and beech, hard maple, cottonwood and aspen among the broad-leaf trees. Traaen's work was done in Norway and related particularly to the Norway spruce *(Picea Abies)*. He found that the spruce was injured when the leaves were subjected to the $CaCl_2$ dust for a period of five to six days. Rainfall seemed to offset the damage, indicating that injury was reduced when the dust was washed from the leaves. The injured needles contained higher amounts of chloride than did the healthy ones.

In any consideration of salt tolerance, it should be borne in mind that the effect of saline substrates may be cumulative and that, over a period of years, even low concentrations of salt may result in a slow but progressive decline of a tree crop (96).

CONCLUSION

It is apparent that heightened interest in salinity and related factors which restrict plant growth in arid or irrigated areas has produced notable contributions to the literature of this field in the past two decades. Quantitative information on the salt tolerance of the most important crop plants has been obtained, and the proximal causes of salt injury have, in some cases, been carefully analyzed. Experimental demonstration of the general occurrence of osmotic effects and the more restricted development of specific ion effects has cleared the way for further advances in understanding the nature of salt injury to plants. Still awaiting clarification is the relative importance of factors which contribute to salt tolerance or sensitivity when osmotic effects predominate. It is obvious that when water availability is restricted by salinity, all the factors which regulate or influence water absorption and water loss by plants contribute in some way to the resultant which is termed "salt tolerance." Little has, as yet, been done to evaluate these factors in terms of their contribution to salt tolerance.

The mechanisms whereby chloride or sodium ions are specifically toxic to sensitive species remain unknown. The similarity of toxic levels of accumulation of these ions in widely diverse species suggests a common mechanism or condition which qualitatively distinguishes these species from non-sensitive species. The mechanism of salt toxicity, and the distinguishing features of salt tolerance, appear to be the major tasks for research on salt tolerance of plants in the decade ahead.

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